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(54) Title: CHEMICALLY-MODIFIED HUMAN GROWTH HORMONE CONJUGATES

(57) Abstract: The present invention provides a chemically modified human Growth Hormone (hGH) prepared by binding a water soluble polymer to the protein. The chemically-modified protein according to the present invention may have a much longer lasting hGH activity than that of the unmodified hGH, enabling reduced dose and scheduling opportunities.

CHEMICALLY-MODIFIED HUMAN GROWTH HORMONE CONJUGATES

The present application claims priority under Title
35, United States Code, §119 to United States
Provisional application Serial No. 60/331,907, filed
November 20, 2001, which is incorporated by reference
in their entirety as if written herein.

FIELD OF THE INVENTION

The present invention relates to a chemical
modification of human Growth Hormone (hGH) and agonist
variants thereof by which the chemical and/or
physiological properties of hGH can be changed. The
PEGylated hGH may have an increased plasma residency
duration, decreased clearance rate, improved
stability, decreased antigenicity, or a combination
thereof. The present invention also relates to
processes for the modification of hGH. In addition,
the present invention relates to pharmaceutical
compositions comprising the modified hGH. A further
embodiment is the use of the modified hGH for the
treatment of growth and development disorders.

BACKGROUND OF THE INVENTION

Human growth hormone (hGH) is a protein
comprising a single chain of 191 amino acids cross-
linked by two disulphide bridges and the monomeric
form has a molecular weight of 22 kDa. Human GH is
secreted by the pituitary gland and which also can be
produced by recombinant genetic engineering. hGH will
cause growth in all bodily tissues that are capable of

growth. Recombinant hGH has been commercially available for several years. Two types of therapeutically useful recombinant hGH preparations are present on the market: the authentic one, e.g.

5   Genotropin™, or Nutropin™ and an analogue with an additional methionine residue at the N-terminal end, e.g. Somatonorm™. hGH is used to stimulate linear growth in patients with hypo pituitary dwarfism also referred to as Growth Hormone Deficiency (GHD) or

10 Turner's syndrome but other indications have also been suggested including long-term treatment of growth failure in children who were born short for gestational age (SGA), for treatment of patients with Prader-Willi syndrome (PWS), chronic renal

15 insufficiency (CRI), Aids wasting, and Aging.

A major biological effect of growth hormone (GH) is to promote growth in young mammals and maintenance of tissues in older mammals. The organ systems

20 affected include the skeleton, connective tissue, muscles, and viscera such as liver, intestine, and kidneys. Growth hormones exert their effect through interaction with specific receptors on the target cell's membrane. hGH is a member of a family of

25 homologous hormones that include placental lactogens, prolactins, and other genetic and species variants or growth hormone (Nicoll, C. S., et al. (1986) *Endocrine Reviews* 7: 169). hGH is unusual among these in that it exhibits broad species specificity and binds to either

30 the cloned somatogenic (Leung, D. W., et al. [1987] *Nature* 330; 537) or prolactin receptor (Boutin, J. M., et al. [1988] *Cell*; 53: 69). The cloned gene for hGH has been expressed in a secreted form in Escherichia coli (Chang, C. N., et al. [1987] *Gene* 55:189), and

35 its DNA and amino acid sequence has been reported

(Goeddel, et al. [1979] *Nature* 281: 544; Gray, et al. [1985] *Gene* 39:247).

5   Human growth hormone (hGH) participates in much of the regulation of normal human growth and development. This pituitary hormone exhibits a multitude of biological effects including linear growth (somatogenesis), lactation, activation of macrophages, insulin-like and diabetogenic effects

10   among others (Chawla, R, K. (1983) *Ann. Rev. Med.* 34, 519; Edwards, C. K. et al. (1988) *Science* 239, 769; Thomer, M. O., et al. (1988) *J. Clin. Invest.* 81:745). Growth hormone deficiency in children leads to dwarfism, which has been successfully treated for more

15   than a decade by exogenous administration of hGH.

       Human growth hormone (hGH) is a single-chain polypeptide consisting of 191 amino acids (molecular weight 21,500). Disulfide bonds link positions 53 and

20   165 and positions 182 and 189. Niall, *Nature, New Biology*, 230:90 (1971). hGH is a potent anabolic agent, especially due to retention of nitrogen, phosphorus, potassium, and calcium. Treatment of hypophysectomized rats with GH can restore at least a

25   portion of the growth rate of the rats. Moore et al., *Endocrinology* 122:2920-2926 (1988). Among its most striking effects in hypo pituitary (GH-deficient) subjects is accelerated linear growth of bone-growth-plate-cartilage resulting in increased stature.

30   Kaplan, *Growth Disorders in Children and Adolescents* (Springfield, IL: Charles C. Thomas, 1964).

       hGH causes a variety of physiological and metabolic effects in various animal models including

35   linear bone growth, lactation, activation of

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macrophages, insulin-like and diabetogenic effects, and others (R. K. Chawla et al., *Annu. Rev. Med.* 34:519 (1983); O. G. P. Isaksson et al., *Annu. Rev. Physiol.* 47, 483 (1985); C. K. Edwards et al., *Science*
5 239, 769 (1988); M. O. Thomer and M. L. Vance, *J. Clin. Invest.* 82:745 (1988); J. P. Hughes and H. G. Friesen, *Ann. Rev. Physiol.* 47:469 (1985)). It has been reported that, especially in women after menopause, GH secretion declines with age. Millard et
10 al., *Neurobiol. Aging*, 11:229-235 (1990); Takahashi et al., *Neuroendocrinology* M, L6- 137-142 (1987). See also Rudman et al., *J. Clin. Invest.*, 67:1361-1369 (1981) and Blackman, *Endocrinology and Aging*, 16:981 (1987). Moreover, a report exists that some of the
15 manifestations of aging, including decreased lean body mass, expansion of adipose-tissue mass, and the thinning of the skin, can be reduced by GH treatment three times a week. See, e.g., Rudman et al., *N. Eng. J. Med.*, 323:1-6 (1990) and the accompanying article
20 in the same journal issue by Dr. Vance (pp. 52-54). These biological effects derive from the interaction between hGH and specific cellular receptors. Two different human receptors have been cloned, the hGH liver receptor (D. W. Leung et al., *Nature*
25 330:537(1987)) and the human prolactin receptor (J. M. Boutin et al., *Mol. Endocrinology.* 3:1455 (1989)). However, there are likely to be others including the human placental lactogen receptor (M. Freemark, M. Comer, G. Komer, and S. Handwerger, *Endocrinol.*
30 120:1865 (1987)). These homologous receptors contain a glycosylated extracellular hormone binding domain, a single transmembrane domain, and a cytoplasmic domain, which differs considerably in sequence and size. One or more receptors are assumed to play a determining
35 role in the physiological response to hGH.

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It is generally observed that physiologically active proteins administered into a body can show their pharmacological activity only for a short period of time due to their high clearance rate in the body.
5 Furthermore, the relative hydrophobicity of these proteins may limit their stability and/or solubility.

For the purpose of decreasing the clearance rate, improving stability or abolishing antigenicity of therapeutic proteins, some methods have been proposed
10 wherein the proteins are chemically modified with water-soluble polymers. Chemical modification of this type may block effectively a proteolytic enzyme from physical contact with the protein backbone itself, thus preventing degradation. Chemical attachment of
15 certain water-soluble polymers may effectively reduce renal clearance due to increased hydrodynamic volume of the molecule. Additional advantages include, under certain circumstances, increasing the stability and circulation time of the therapeutic protein,
20 increasing solubility, and decreasing immunogenicity. Poly(alkylene oxide), notably poly(ethylene glycol) (PEG), is one such chemical moiety that has been used in the preparation of therapeutic protein products (the verb "pegylate" meaning to attach at least one
25 PEG molecule). The attachment of poly(ethylene glycol) has been shown to protect against proteolysis, Sada, et al., *J. Fermentation Bioengineering* 71: 137-139 (1991), and methods for attachment of certain poly(ethylene glycol) moieties are available. See U.S.
30 Pat. No. 4,179,337, Davis et al., "Non-Immunogenic Polypeptides," issued Dec. 18, 1979; and U.S. Pat. No. 4,002,531, Royer, "Modifying enzymes with Polyethylene Glycol and Product Produced Thereby," issued Jan. 11, 1977. For a review, see Abuchowski et al., in Enzymes

as Drugs. (J. S. Holcerberg and J. Roberts, eds. pp. 367-383 (1981)).

Other water-soluble polymers have been used, such as copolymers of ethylene glycol/propylene glycol,
5   carboxymethylcellulose, dextran, poly(vinyl alcohol), poly(vinyl pyrrolidone), poly(-1,3-dioxolane), poly(-1,3,6-trioxane), ethylene/maleic anhydride copolymer, poly- amino acids (either homopolymers or random copolymers).
10

A number of examples of pegylated therapeutic proteins have been described. ADAGEN®, a pegylated formulation of adenosine deaminase, is approved for treating severe combined immunodeficiency disease.
15   ONCASPAR®, a pegylated L-asparaginase has been approved for treating hypersensitive ALL patients. Pegylated superoxide dismutase has been in clinical trials for treating head injury. Pegylated α-interferon (U.S. 5,738,846, 5,382,657) has been
20   approved for treating hepatitis; pegylated glucocerebrosidase and pegylated hemoglobin are reported to have been in preclinical testing. Another example is pegylated IL-6, EP 0 442 724, entitled, "Modified hIL-6," which discloses poly(ethylene
25   glycol) molecules added to IL-6.
Another specific therapeutic protein, which has been chemically modified, is granulocyte colony stimulating factor, (G-CSF). G-CSF induces the rapid proliferation and release of neutrophilic granulocytes
30   to the blood stream, and thereby provides therapeutic effect in fighting infection. European patent publication EP 0 401 384, published Dec. 12, 1990, entitled, "Chemically Modified Granulocyte Colony Stimulating Factor," describes materials and methods
35   for preparing G-CSF to which poly(ethylene glycol)

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molecules are attached. Modified G-CSF and analogs
thereof are also reported in EP 0 473 268, published
Mar. 4, 1992, entitled "Continuous Release
Pharmaceutical Compositions Comprising a Polypeptide

5   Covaléntly Conjugated To A Water Soluble Polymer,"
stating the use of various G-CSF and derivatives
covalently conjugated to a water soluble particle
polymer, such as poly(ethylene glycol). A modified
polypeptide having human granulocyte colony

10   stimulating factor activity is reported in EP 0 335
423 published Oct. 4, 1989. Provided in U.S. 5,824,784
are methods for N-terminally modifying proteins or
analogs thereof, and resultant compositions, including
novel N-terminally chemically modified G-CSF

15   compositions. U.S. 5,824,778 discloses chemically
modified G-CSF.

        For poly(ethylene glycol), a variety of means
have been used to attach the poly(ethylene glycol)
molecules to the protein. Generally, poly(ethylene

20   glycol) molecules are connected to the protein via a
reactive group found on the protein.

        Amino groups, such as those on lysine residues or
at the N-terminus, are convenient for such attachment.
For example, Royer (U.S. Pat. No. 4,002,531, above)

25   states that reductive alkylation was used for
attachment of poly(ethylene glycol) molecules to an
enzyme. EP 0 539 167, published Apr. 28, 1993, Wright,
"Peg Imidates and Protein Derivatives Thereof" states
that peptides and organic compounds with free amino

30   group(s) are modified with an imidate derivative of
PEG or related water-soluble organic polymers. US
5,298,643 and US 5,637,749 disclose PEG aryl imidates

        Chamow et al., Bioconjugate Chem. 5: 133-140
(1994) report the modification of CD4 immunoadhesin

35   with monomethoxypoly(ethylene glycol) aldehyde via

reductive alkylation. The authors report that 50% of
the CD4-Ig was MePEG-modified under conditions
allowing control over the extent of pegylation. *Id.* at
page 137. The authors also report that the *in vitro*
5 binding capability of the modified CD4-Ig (to the
protein gp 120) decreased at a rate correlated to the
extent of MePEGylation *Ibid.* U.S. Pat. No. 4,904,584,
Shaw, issued Feb. 27, 1990, relates to the
modification of the number of lysine residues in
10 proteins for the attachment of poly(ethylene glycol)
molecules via reactive amine groups.

Many methods of attaching a polymer to a protein
involve using a moiety to act as a linking group. Such
moieties may, however, be antigenic. A tresyl
15 chloride method involving no linking group is
available, but this method may be difficult to use to
produce therapeutic products as the use of tresyl
chloride may produce toxic by-products. See Francis *et
al.*, In: Stability of protein pharmaceuticals: in vivo
20 pathways of degradation and strategies for protein
stabilization (Eds. Ahern, T. and Manning, M. C.)
Plenum, New York, 1991) Also, Delgado *et al.*,
"Coupling of PEG to Protein By Activation With Tresyl
Chloride, Applications In Immunoaffinity Cell
25 Preparation", in Separations Using Aqueous Phase
Systems, Applications In Cell Biology and
Biotechnology, Fisher *et al.*, eds. Plenum Press, New
York, N.Y., 1989 pp. 211-213.

See also, Rose *et al.*, *Bioconjugate Chemistry* 2:
30 154-159 (1991) which reports the selective attachment
of the linker group carbohydrazide to the C-terminal
carboxyl group of a protein substrate (insulin).

WO 93/00109 relates to a method for stimulating a
mammal's or avian's GH responsive tissues comprising
35 maintaining a continuous, effective plasma GH

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concentration for a period of 3 or more days. One way
of achieving such plasma concentration is stated to be
by use of GH coupled to a macromolecular substance
such as PEG (polyethylene glycol). The coupling to a
5   macromolecular substance is stated to result in
improved half-life. PEGylated human growth hormone has
been reported in WO 93/00109 using mPEG aldehyde-5000
and mPEG N-hydroxysuccinmidyl ester(mPEG-NHS-5000).
The use of mPEG-NHS resulted in heterogeneous mixtures
10   of multiply PEGylated forms of hGH. WO 93/00109 also
discloses the use of mPEG-maleimide to PEGylate
cysteine hGH variants.

WO 99/03887 discloses a cysteine variant growth
15   hormone that is PEGylated. Designated as BT-005, this
conjugate is purported to be more effective at
stimulating weight gain in growth hormone deficient
rats and to have a longer half-life than hGH.
PEGylated human growth hormone has also been
20   reported in Clark et al. using succinimidyl ester of
carboxymethylated PEG (*Journal of Biological Chemistry*
271:21969-21977, 1996). Clark et al. describes
derivates of hGH of increasing size using mPEG-NHS-
5000, which selectively conjugates to primary amines.
25   Increasing levels of PEG modification reduced the
affinity for its receptor and increased the EC_{50} in a
cell-based assay up to 1500 fold. Olson et al.,
Polymer Preprints 38:568-569, 1997 discloses the use
of N-hydroxysuccinimide (NHS)PEG and succinimidyl
30   propionate (SPA)PEG to achieve multiply PEGylated hGH
species.
WO 94/20069 prophetically discloses PEGylated hGH
as part of a formulation for pulmonary delivery.

35   US 4,179,337 discloses methods of PEGylating

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enzymes and hormones to obtain physiologically active
non-immunogenic, water-soluble polypeptide conjugates.
GH is mentioned as one example of a hormone to be
PEGylated.

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EP 458064 A2 discloses PEGylation of introduced
or naturally present cysteine residues in
somatotropin. EP 458064 A2 further mentions the
incorporation of two cysteine residues in a loop

10   termed the omega loop stated to be located at residues
102-112 in wild type bovine somatotropin, more
specifically EP 458064 A2 discloses the substitution
of residues numbered 102 and 112 of bovine
somatotropin from Ser to Cys and Tyr to Cys,

15   respectively.

WO 95/11987 suggests attachment of PEG to the
thiol group of a cysteine residue being either present
in the parent molecule or introduced by site directed

20   mutagenesis. WO 95/11987 relates to PEGylation of
protease nexin-1, however PEGylation in general of hGH
and other proteins is suggested as well.

WO 99/03887 discloses, e.g., growth hormone

25   modified by insertion of additional cysteine for
serine residues and attachment of PEG to the
introduced cysteine residues.

WO 00/42175 relates to a method for making

30   proteins containing free cysteine residues for
attachment of PEG. WO 00/42175 discloses the following
muteins of hGH: T3C, S144C and T148C and the cysteine
PEGylation thereof.

35   WO 9711178 (as well as US 5849535, US 6004931,

and US 6022711) relates to the use of GH variants as agonists or antagonists of hGH. WO 9711178 also discloses PEGylation of hGH, including lysine PEGylation and the introduction or replacement of

5   lysine (e.g. K168A and K172R). WO 9711178 also discloses the substitution G120K.

The previous reports of PEGylated hGH require the attachment of multiple PEGs, which results in

10   undesirable product heterogeneity, to achieve a hydrodynamic volume greater than the 70K molecular weight cut-off of the kidney filtration as described (Knauf, M.J. et al, *J. Biol. Chem.* 263:15064-15070, 1988).

15

A GH molecule with a longer circulation half-life would decrease the number of necessary administrations and potentially provide more optimal therapeutic hGH levels with concomitant enhanced therapeutic effect.

20

The present invention provides chemically modified hGH conjugates having decreased heterogeneity, decreased clearance rate, increased plasma residency duration, improved solubility,

25   increased stability, decreased antigenicity, or combinations thereof.

SUMMARY OF THE INVENTION

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The present invention relates to chemically modified hGH and agonist variants thereof, which have at least one improved chemical or physiological property selected from but not limited to decreased

35   clearance rate, increased plasma residency duration,

increased stability, improved solubility, and decreased antigenicity. Thus, as described below in more detail, the present invention has a number of aspects relating to chemically modifying hGH and

5   agonist variants thereof as well as specific modifications using a variety of poly(ethylene glycol) moieties.

The present invention also relates to methods of producing the chemically modified hGH and agonist

10   variants thereof.

The present invention also relates to compositions comprising the chemically modified hGH and agonist variants thereof.

The modified hGH and agonist variants thereof of

15   the present invention may be useful in the treatment of, but not limited to, dwarfism (GHD), Adult GHD, Turner's syndrome, long-term treatment of growth failure in children who were born short for gestational age (SGA), for treatment of patients with

20   Prader-Willi syndrome (PWS), chronic renal insufficiency (CRI); Aids wasting, Aging, End-stage Renal Failure, and Cystic Fibrosis.

BRIEF DESCRIPTION OF THE DRAWINGS

25

Figure 1 is a reproduction of a reducing and non-reducing SDS-PAGE analysis of the products of the reaction of hGH and 20K PEG-ALD and the anion exchange purified 20K PEG-ALD hGH. Lane 1. MW Protein

30   standards; Lane 2. reduced hGH-10 ug; Lane 3. reduced 20 K linear PEG-ALD hGH reaction mix-10 ug; Lane 4. reduced anion exchange purified 20 K linear PEG-ALD hGH-10 ug Lane 5. Blank; Lane 6. non-reduced hGH-10 ug; Lane 7. non-reduced 20 K linear PEG-ALD hGH

35   reaction mix-10 ug; Lane 8. non-reduced anion

exchange purified 20 K linear PEG-ALD hGH-10 ug; Lane
9. Blank; Lane 10. MW Protein standards.

Figure 2 is a reproduction of a non-reducing SDS-PAGE
5   analysis of various anion exchange purified pegylated
hGH molecules. Lane 1. MW Protein standards; Lane 2.
hGH-10 ug; Lane 2. 4-6 x 5K PEG-SPA hGH-10 ug; Lane 3.
20 K linear PEG-ALD hGH-10 ug; Lane 4. 20 K branched
PEG-ALD hGH-10 ug Lane 5. 40 K branched PEG hGH-10
10 ug.

Figure 3 shows reproductions of RP-HPLC elution
profiles for trypsin digests of hGH, 40K Br PEG-ALD
hGH and 40K Br PEG-NHS hGH. PEG coupled primarily to
15 the N-terminus of hGH (as shown in the 40K Br ALD hGH)
results in a reduction in the N-terminal (T1) fragment
peak with generation of a new PEGylated T1 peak.

Figure 4 compares the *in vivo* bioactivity of
20 unPEGylated hGH dosed daily (0.3 mg/Kg/day) to mono-
PEGylated hGH dosed subcutaneously(SC) once every six
days(1.8 mg/Kg) by illustrating the weight gain in
hypophysectomized rats during a period of 11 days.

25 Figure 5 compares the *in vivo* bioactivity of
unPEGylated hGH dosed SC daily (0.3 mg/Kg/day) to 4-6
x 5K PEG-SPA-hGH, mono-PEGylated 20K branched PEG-ALD
hGH, and mono-PEGylated 40K branched PEG-ALD hGH each
dosed SC once every six days (1.8 mg/Kg) by
30 illustrating the weight gain in hypophysectomized rats
during a period of 11 days.

Figure 6 compares the *in vivo* bioactivity of
unPEGylated hGH dosed SC daily (0.3 mg/Kg/day) to 4-6
35 x 5K PEG-CMHBA-hGH, mono-PEGylated 20K linear ALD,

mono-PEGylated 30K linear ALD, mono-PEGylated 20K
branched PEG-ALD hGH, and mono-PEGylated 40K branched
PEG-ALD hGH each dosed SC once every six days (1.8
mg/Kg) by illustrating the increase in tibial bone
5   growth in hypophysectomized rats during a period of 11
days.

Figure 7 compares the *in vivo* bioactivity of a single
1.8 mg/Kg SC dose of unPEGylated hGH, mono-PEGylated
10   5K linear PEG-ALD hGH, mono-PEGylated 20K linear PEG-
ALD hGH, mono-PEGylated 20K branched PEG-ALD hGH,
mono-PEGylated 20K linear PEG-Hydrazide hGH, mono-
PEGylated 30K linear PEG-ALD hGH, mono-PEGylated 40K
branched PEG-ALD hGH, 4-6 x 5K PEG SPA hGH, 4-6 x 5K
15   PEG-CMHBA hGH by illustrating the increase in plasma
IGF-1 levels in hypophysectomized rats during a period
of 9 days.

DETAILED DESCRIPTION

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hGH and agonist variants thereof are members of a
family of recombinant proteins, described in US
4,658,021 and US 5,633,352. Their recombinant
production and methods of use are detailed in US
25   4,342,832, 4,601,980; US 4,898,830; US 5,424,199; and
US 5,795,745.
Any purified and isolated hGH or agonist variant
thereof, which is produced by host cells such as *E.
coli* and animal cells transformed or transfected by
30   using recombinant genetic techniques, may be used in
the present invention. Additional hGH variants are
described in U.S. Ser. No. 07/715,300 filed Jun.14,
1991 and Ser. No. 07/743,614 filed Aug. 9, 1991, and
WO 92/09690 published Jun. 11, 1992. Among them, hGH
35   or agonist variant thereof, which is produced by the

transformed *E. coli*, is particularly preferable. Such hGH or agonist variant thereof may be obtained in large quantities with high purity and homogeneity. For example, the above hGH or agonist variant thereof may

5   be prepared according to a method disclosed in US 4,342,832, 4,601,980; US 4,898,830; US 5,424,199; and US 5,795,745. The term "substantially has the following amino acid sequence" means that the above amino acid sequence may include one or more amino-acid

10   changes (deletion, addition, insertion or replacement) as long as such changes will not cause any disadvantageous non-similarity in function to hGH or agonist variant thereof. It is more preferable to use the hGH or agonist variant thereof substantially

15   having an amino acid sequence, in which at least one lysine, aspartic acid, glutamic acid, unpaired cysteine residue, a free N-terminal α-amino group or a free C-terminal carboxyl group, is included.

According to the present invention, poly(ethylene

20   glycol) is covalently bound through amino acid residues of hGH or agonist variant thereof. A variety of activated poly(ethylene glycol)s having a number of different functional groups, linkers, configurations, and molecular weights are known to one skilled in the

25   art, which may be used to create PEG-hGH conjugates or PEG-hGH agonist variant conjugates (for reviews see Roberts M.J. et al., *Adv. Drug Del. Rev.* 54:459-476, 2002), Harris J.M. et al., *Drug Delivery Sytems* 40:538-551, 2001) The amino acid residue may be any

30   reactive one(s) having, for example, free amino, carboxyl, sulfhydryl (thiol), hydroxyl, guanidinyl, or imidizoyl groups, to which a terminal reactive group of an activated poly(ethylene glycol) may be bound. The amino acid residues having the free amino groups

35   may include lysine residues and/or N-terminal amino

acid residue, those having a free carboxyl group may
include aspartic acid, glutamic acid and/or C-terminal
amino acid residues, those having a free sulfhydryl
(thiol) such as cysteine, those having a free hydroxyl
5   such as serine or tyrosine, those having a free
guanidinyl such as arginine, and those having a free
imidizoyl such as histidine.

In another embodiment, oxime chemistries (Lemieux
& Bertozzi *Tib Tech* **16**:506-513, 1998) are used to
10 target N-terminal serine residues.

The poly(ethylene glycol) used in the present
invention is not restricted to any particular form or
molecular weight range. The poly(ethylene glycol)
molecular weight may between 500 and 100,000.
15 Normally, a molecular weight of 500-60,000 is used and
preferably of from 1,000-40,000. More preferable, the
molecular weight is greater than 5,000 to about
40,000.

In another embodiment the poly(ethylene glycol)
20 is a branched PEG having more than one PEG moiety
attached. Preferred examples of branched PEGs are
described in U.S. 5,932,462; U.S. 5,342,940; U.S.
5,643,575; U.S. 5,919,455; U.S. 6,113,906; U.S.
5,183,660; WO 02/09766; Kodera Y., *Bioconjugate*
25 *Chemistry* 5:283-288 (1994); and Yamasaki et al.,
Agric. Biol. Chem., 52:2125-2127, 1998. In a preferred
embodiment the molecular weight of each poly(ethylene
glycol) of the branched PEG is 5,000-20,000.

Poly(alkylene oxide)s, notably poly(ethylene
30 glycol)s, are bound to hGH or agonist variant thereof
via a terminal reactive group, which may or may not
leave a linking moiety (spacer) between the PEG and
the protein. In order to form the hGH conjugates or
agonist variant thereof of the present invention,
35 polymers such as poly(alkylene oxide) are converted

into activated forms, as such term is known to those
of ordinary skill in the art. The reactive group, for
example, is a terminal reactive group, which mediates
a bond between chemical moieties on the protein, such
5   as amino, carboxyl or thiol groups, and poly(ethylene
glycol). Typically, one or both of the terminal
polymer hydroxyl end-groups, (i.e. the alpha and omega
terminal hydroxyl groups) are converted into reactive
functional groups, which allows covalent conjugation.
10   This process is frequently referred to as "activation"
and the poly(ethylene glycol) product having the
reactive group is hereinafter referred to as "an
activated poly(ethylene glycol)". Polymers containing
both α and ε linking groups are referred to as "bis-
15   activated poly(alkylene oxides)" and are referred to
as "bifunctional". Polymers containing the same
reactive group on α and ε terminal hydroxyls are
sometimes referred to as "homobifunctional" or
"homobis-activated". Polymers containing different
20   reactive groups on α and ε terminal hydroxyls are
sometimes referred to as "heterobifunctional" (see for
example WO 01/26692) or "heterobis-activated".
Polymers containing a single reactive group are
referred to as "mono-activated" polyalkylene oxides or
25   "mono-functional". Other substantially non-antigenic
polymers are similarly "activated" or
"functionalized".

    The activated polymers are thus suitable for
mediating a bond between chemical moieties on the
30   protein, such as α- or ε-amino, carboxyl or thiol
groups, and poly(ethylene glycol). Bis-activated
polymers can react in this manner with two protein
molecules or one protein molecule and a reactive small
molecule in another embodiment to effectively form

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protein polymers or protein-small molecule conjugates through cross linkages.

Functional groups capable of reacting with either the amino terminal α-amino group or ε-amino groups of

5   lysines found on the hGH or agonist variant thereof include: N-hydroxysuccinimidyl esters, carbonates such as the p-nitrophenyl, or succinimidyl (US 5,808,096, 5,612,460, US 5,324,844, US 5,5,122,614); carbonyl imidazole; azlactones (US 5,321,095, US 5,567,422);

10 cyclic imide thiones (US 5,405,877, 5,349,001); isocyanates or isothiocyanates (Greenwald R.B., *J. Org. Chem.*, 60:331-336, 1995); tresyl chloride (EP 714 402, EP 439 508); halogen formiates (WO 96/40792), and aldehydes.

15 Functional groups capable of reacting with carboxylic acid groups, reactive carbonyl groups and oxidized carbohydrate moieties on hGH or agonist variant thereof include; primary amines; and hydrazine and hydrazide functional groups such as the acyl

20 hydrazides, carbazates, semicarbamates, thiocarbazates, etc (WO 01/70685).

Mercapto groups, if available on the hGH or agonist variant thereof, can also be used as attachment sites for suitably activated polymers with

25 reactive groups such as thiols; maleimides, sulfones, and phenyl glyoxals; see, for example, U.S. Pat. No. 5,093,531, the disclosure of which is hereby incorporated by reference. Other nucleophiles capable of reacting with an electrophilic center include, but

30 are not limited to, for example, hydroxyl, amino, carboxyl, thiol, active methylene and the like.

Also included are polymers including lipophilic and hydrophilic moieties disclosed in US 5,359,030 and US 5,681,811; US 5,438,040; and US 5,359,030.

As well halogenated PEGs are disclosed on WO
98/32466 that can react with amino, thiol groups, and
aromatic hydroxy groups, which directly covalently
attach the PEG to the protein.

5   In one preferred embodiment of the invention secondary
amine or amide linkages are formed using the N-
terminal α-amino group or ε-amino groups of lysine of
hGH or agonist variant thereof and the activated PEG.
In another preferred aspect of the invention, a

10   secondary amine linkage is formed between the N-
terminal primary α- or ε-amino group of hGH or agonist
variant thereof and single or branched chain PEG
aldehyde by reduction with a suitable reducing agent
such as $NaCNBH_3$, $NaBH_3$, Pyridine Borane etc. as

15   described in Chamow et al., Bioconjugate Chem. 5: 133-
140 (1994) and US Pat. No 5,824,784.

In a preferred embodiment at least 70%,
preferably at least 80%, preferably at least 81%,
preferably at least 82%, preferably at least 83%,

20   preferably at least 84%, preferably at least 85%,
preferably at least 86%, preferably at least 87%,
preferably at least 88%, preferably at least 89%,
preferably at least 90%, preferably at least 91%,
preferably at least 92%, preferably at least 93%,

25   preferably at least 94%, preferably at least 95%,
preferably at least 96%, preferably at least 97%, and
most preferably at least 98% of the poly(ethylene
glycol) is on the amino terminal α-amino group.

In another preferred embodiment of the invention,

30   polymers activated with amide-forming linkers such as
succinimidyl esters, cyclic imide thiones, or the like
are used to effect the linkage between the hGH or
agonist variant thereof and polymer, see for example,
U.S. Pat. No. 5,349,001; U.S. Pat. No. 5,405,877; and

35   Greenwald, et al., Crit. Rev. Ther. Drug Carrier Syst.

17:101-161, 2000, which are incorporated herein by reference. One preferred activated poly(ethylene glycol), which may be bound to the free amino groups of hGH or agonist variant thereof includes single or

5   branched chain N-hydroxysuccinylimide poly(ethylene glycol) may be prepared by activating succinic acid esters of poly(ethylene glycol) with N-hydroxysuccinylimide.

Other preferred embodiments of the invention
10   include using other activated polymers to form covalent linkages of the polymer with the hGH or agonist variant thereof via ε-amino or other groups. For example, isocyanate or isothiocyanate forms of terminally activated polymers can be used to form urea
15   or thiourea-based linkages with the lysine amino groups (Greenwald R.B., *J. Org. Chem.*, 60:331-336, 1995).

In another preferred aspect of the invention, carbamate (urethane) linkages are formed with protein
20   amino groups as described in U.S. Pat. Nos. 5,122,614, 5,324,844, and 5,612,640, which are hereby incorporated by reference. Examples include N-succinimidyl carbonate, para-nitrophenyl carbonate, and carbonyl imidazole activated polymers. In another
25   preferred embodiment of this invention, a benzotriazole carbonate derivative of PEG is linked to amino groups on hGH or agonist variant thereof.

Another aspect of the invention represents a prodrug or sustained release form of hGH or agonist
30   variant thereof, comprised of a water soluble polymer, such as poly(ethylene glycol), attached to an hGH or agonist variant thereof molecule by a functional linker that can predictably break down by enzymatic or pH directed hydrolysis to release free hGH or agonist
35   variant thereof or other hGH or agonist variant

thereof derivative. The prodrug can also be a "double
prodrug" (Bundgaard in *Advanced Drug Delivery Reviews*
3:39-65, 1989) involving the use of a cascade
latentiation. In such systems, the hydrolytic reaction
5 involves an initial rate-limiting (slow) enzymatic or
pH directed step and a second step involving a rapid
non-enzymatic hydrolysis that occurs only after the
first has taken place. Such a releasable polymer
provides protein conjugates, which are impermanent and
10 could act as a reservoir, that continually discharge
hGH or agonist variant thereof. Such functional
linkers are described in US 5,614,549; US 5,840,900;
US 5,880,131; US 5,965,119; us 5,965,565; US
6,011,042; US 6,153,655; US 6,180,095 B1; US
15 6,413,507; Greenwald R.B. et al., *J. Med. Chem.*
42;3657-3667, 1999; Lee, S. et al., *Bioconjugate Chem*
12:163-169, 2001; Garman A.J. et al., *FEBS Lett.*
223:361-365, 1987; Woghiren C. et al., *Bioconjucate*
Chem. 4:314-318, 1993; Roberts M.J. et al., *J. Pharm.*
20 *Sci. 87*;1440-1445, 1998; Zhao X., in *Ninth Int. Symp.*
Recent Adv. Drug Delivery Syst. 199; Greenwald R.B. et
al., *J. Med. Chem. 43*:475-467, 2000; and Greenwald
R.B. *Crit. Rev. Ther. Drug Carrier Syst. 17*:101-161,
2000. Zalipsky et al., 28th Int. Symp. On controlled
25 Release of Bioactive Materials 1; 73-74,2001

Conjugation reactions, referred to as pegylation
reactions, were historically carried out in solution
with molar excess of polymer and without regard to
30 where the polymer will attach to the protein. Such
general techniques, however, have typically been
proven inadequate for conjugating bioactive proteins
to non-antigenic polymers while retaining sufficient
bioactivity. One way to maintain the hGH or agonist

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variant thereof bioactivity is to substantially avoid
the conjugation of those hGH or agonist variant
thereof reactive groups associated with the receptor
binding site(s) in the polymer coupling process.

5   Another aspect of the present invention is to provide
a process of conjugating poly(ethylene glycol) to hGH
or agonist variant thereof maintaining high levels of
retained activity.

       The chemical modification through a covalent
10   bond may be performed under any suitable condition
generally adopted in a reaction of a biologically
active substance with the activated poly(ethylene
glycol). The conjugation reaction is carried out under
relatively mild conditions to avoid inactivating the
15   hGH or agonist variant thereof. Mild conditions
include maintaining the pH of the reaction solution in
the range of 3 to 10 and the reaction temperatures
within the range of from about 0°-37°C. In the cases
where the reactive amino acid residues in hGH or
20   agonist variant thereof have free amino groups, the
above modification is preferably carried out in a non-
limiting list of suitable buffers (pH 3 to 10),
including phosphate, MES, citrate, acetate, succinate
or HEPES, for 1-48 hrs at 4°-37°C. In targeting N-
25   terminal amino groups with reagents such as PEG
aldehydes pH 4-8 is preferably maintained. The
activated poly(ethylene glycol) may be used in about
0.05-100 times, preferably about 0.01 -2.5 times, the
molar amount of the number of free amino groups of hGH
30   or agonist variant thereof. On the other hand, where
reactive amino acid residues in hGH or agonist variant
thereof have the free carboxyl groups, the above
modification is preferably carried out in pH from
about 3.5 to about 5.5, for example, the modification
35   with poly(oxyethylenediamine) is carried out in the

presence of carbodiimide (pH 3.5-5) for 1-24 hrs at 4°
-37°C. The activated poly(ethylene glycol) may be used
in 0.05-300 times the molar amount of the number of
free carboxyl groups of hGH or agonist variant

5   thereof.

In separate embodiments, the upper limit for the
amount of polymer included in the conjugation
reactions exceeds about 1:1 to the extent that it is
possible to react the activated polymer and hGH or

10   agonist variant thereof without forming a substantial
amount of high molecular weight species, i.e. more
than about 20% of the conjugates containing more than
about one strand of polymer per molecule of hGH or
agonist variant thereof. For example, it is

15   contemplated in this aspect of the invention that
ratios of up to about 6:1 can be employed to form
significant amounts of the desired conjugates which
can thereafter be isolated from any high molecular
weight species.

20   In another aspect of this invention,
bifunctionally activated PEG derivatives may be used
to generate polymeric hGH or agonist variant thereof-
PEG molecules in which multiple hGH or agonist variant
thereof molecules are crosslinked via PEG. Although

25   the reaction conditions described herein can result in
significant amounts of unmodified hGH or agonist
variant thereof, the unmodified hGH or agonist variant
thereof can be readily recycled into future batches
for additional conjugation reactions. The processes of

30   the present invention generate surprisingly very
little, i.e. less than about 30% and more preferably,
less than about 10%, of high molecular weight species
and species containing more than one polymer strand
per hGH or agonist variant thereof. These reaction

35   conditions are to be contrasted with those typically

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used for polymeric conjugation reactions wherein the activated polymer is present in several-fold molar excesses with respect to the target. In other aspects of the invention, the polymer is present in amounts of

5   from about 0.1/amino group to about 50 equivalents per equivalent of hGH or agonist variant thereof. In other aspects of the invention, the polymer is present in amounts of from about 1 to about 10 equivalents per equivalent of hGH or agonist variant thereof.

10   The conjugation reactions of the present invention initially provide a reaction mixture or pool containing mono- and di-PEG-hGH conjugates, unreacted hGH, unreacted polymer, and usually less than about 20% high molecular weight species. The high molecular

15   weight species include conjugates containing more than one polymer strand and/or polymerized PEG-hGH or agonist variant thereof species. After the unreacted species and high molecular weight species have been removed, compositions containing primarily mono- and

20   di-polymer-hGH or agonist variant thereof conjugates are recovered. Given the fact that the conjugates for the most part include a single polymer strand, the conjugates are substantially homogeneous. These modified hGH or agonist variant thereof have at least

25   about 0.1% of the *in vitro* biological activity associated with the native or unmodified hGH or agonist variant thereof as measured using standard FDC-P1 cell proliferation assays, (Clark et al. *Journal of Biological Chemistry* 271:21969-21977,

30   1996), receptor binding assay (US 5,057,417), or hypophysectomized rat growth (Clark et al. *Journal of Biological Chemistry* 271:21969-21977, 1996). In preferred aspects of the invention, however, the modified hGH or agonist variant thereof have about 25%

35   of the *in vitro* biological activity, more preferably,

24

the modified hGH or agonist variant thereof have about
50% of the *in vitro* biological activity, more
preferably, the modified hGH or agonist variant
thereof have about 75% of the *in vitro* biological
5   activity, and most preferably the modified hGH or
agonist variant thereof have equivalent or improved *in
vitro* biological activity.

    The processes of the present invention preferably
include rather limited ratios of polymer to hGH or
10 agonist variant thereof. Thus, the hGH or agonist
variant thereof conjugates have been found to be
predominantly limited to species containing only one
strand of polymer. Furthermore, the attachment of the
polymer to the hGH or agonist variant thereof reactive
15 groups is substantially less random than when higher
molar excesses of polymer linker are used. The
unmodified hGH or agonist variant thereof present in
the reaction pool, after the conjugation reaction has
been quenched, can be recycled into future reactions
20 using ion exchange or size exclusion chromatography or
similar separation techniques.

    A poly(ethylene glycol)-modified hGH or agonist
variant thereof, namely chemically modified protein
according to the present invention, may be purified
25 from a reaction mixture by conventional methods which
are used for purification of proteins, such as
dialysis, salting-out, ultrafiltration, ion-exchange
chromatography, hydrophobic interaction chromatography
(HIC), gel chromatography and electrophoresis. Ion-
30 exchange chromatography is particularly effective in
removing unreacted poly(ethylene glycol) and hGH or
agonist variant thereof. In a further embodiment of
the invention, the mono- and di-polymer-hGH or agonist
variant thereof species are isolated from the reaction
35 mixture to remove high molecular weight species, and

25

unmodified hGH or agonist variant thereof. Separation
is effected by placing the mixed species in a buffer
solution containing from about 0.5-10 mg/mL of the hGH
or agonist variant thereof-polymer conjugates.

5   Suitable solutions have a pH from about 4 to about 8.
The solutions preferably contain one or more buffer
salts selected from KCl, NaCl, K_2HPO_4, KH_2PO_4, Na_2HPO_4,
NaH_2PO_4, $NaHCO_3$, $NaBO_4$, CH_3CO_2H, and NaOH.

Depending upon the reaction buffer, the hGH or
10   agonist variant thereof polymer conjugate solution may
first have to undergo buffer exchange/ultrafiltration
to remove any unreacted polymer. For example, the PEG-
hGH or agonist variant thereof conjugate solution can
be ultrafiltered across a low molecular weight cut-off
15   (10,000 to 30,000 Dalton) membrane to remove most
unwanted materials such as unreacted polymer,
surfactants, if present, or the like.

The fractionation of the conjugates into a pool
containing the desired species is preferably carried
20   out using an ion exchange chromatography medium. Such
media are capable of selectively binding PEG-hGH or
agonist variant thereof conjugates via differences in
charge, which vary in a somewhat predictable fashion.
For example, the surface charge of hGH or agonist
25   variant thereof is determined by the number of
available charged groups on the surface of the
protein. These charged groups typically serve as the
point of potential attachment of poly(alkylene oxide)
polymers. Therefore, hGH or agonist variant thereof
30   conjugates will have a different charge from the other
species to allow selective isolation.

Strongly polar anion or cation exchange resins
such as quaternary amine or sulfopropyl resins,
respectively, are used for the method of the present
35   invention. Ion exchange resins are especially

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preferred. A non-limiting list of included
commercially available cation exchange resins suitable
for use with the present invention are SP-hitrap®, SP
Sepharose HP® and SP Sepharose® fast flow. Other

5   suitable cation exchange resins e.g. S and CM resins
can also be used. A non-limiting list of anion
exchange resins, including commercially available
anion exchange resins, suitable for use with the
present invention are Q-hitrap®, Q Sepharose HP®, and

10 Q sepharose® fast flow. Other suitable anion exchange
resins, e.g. DEAE resins, can also be used.

    For example, the anion or cation exchange resin
is preferably packed in a column and equilibrated by
conventional means. A buffer having the same pH and

15 osmolality as the polymer conjugated hGH or agonist
variant thereof solution is used. The elution buffer
preferably contains one or more salts selected from
KCl, NaCl, K_2HPO_4, KH_2PO_4, Na_2HPO_4, NaH_2PO_4, $NaHCO_3$,
$NaBO_4$, and $(NH_4)_2CO_3$. The conjugate-containing solution

20 is then adsorbed onto the column with unreacted
polymer and some high molecular weight species not
being retained. At the completion of the loading, a
gradient flow of an elution buffer with increasing
salt concentrations is applied to the column to elute

25 the desired fraction of polyalkylene oxide-conjugated
hGH or agonist variant thereof. The eluted pooled
fractions are preferably limited to uniform polymer
conjugates after the cation or anion exchange
separation step. Any unconjugated hGH or agonist

30 variant thereof species can then be back washed from
the column by conventional techniques. If desired,
mono and multiply pegylated hGH or agonist variant
thereof species can be further separated from each

other via additional ion exchange chromatography or size exclusion chromatography.

Techniques utilizing multiple isocratic steps of increasing concentration of salt or pH can also be
5 used. Multiple isocratic elution steps of increasing concentration will result in the sequential elution of di- and then mono-hGH or agonist variant thereof-polymer conjugates.

The temperature range for elution is between
10 about 4°C and about 25°C. Preferably, elution is carried out at a temperature of from about 4°C to about 22°C. For example, the elution of the PEG-hGH or agonist variant thereof fraction is detected by UV absorbance at 280 nm. Fraction collection may be
15 achieved through simple time elution profiles.

A surfactant can be used in the processes of conjugating the poly(ethylene glycol) polymer with the hGH or agonist variant thereof moiety. Suitable surfactants include ionic-type agents such as sodium
20 dodecyl sulfate (SDS). Other ionic surfactants such as lithium dodecyl sulfate, quaternary ammonium compounds, taurocholic acid, caprylic acid, decane sulfonic acid, etc. can also be used. Non-ionic surfactants can also be used. For example, materials
25 such as poly(oxyethylene) sorbitans (Tweens), poly(oxyethylene) ethers (Tritons) can be used. See also Neugebauer, A Guide to the Properties and Uses of Detergents in Biology and Biochemistry (1992) Calbiochem Corp. The only limitations on the
30 surfactants used in the processes of the invention are that they are used under conditions and at concentrations that do not cause substantial irreversible denaturation of the hGH or agonist variant thereof and do not completely inhibit polymer
35 conjugation. The surfactants are present in the

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reaction mixtures in amounts from about 0.01-0.5%;
preferably from 0.05-0.5%; and most preferably from
about 0.075-0.25%. Mixtures of the surfactants are
also contemplated.

5   It is thought that the surfactants provide a
temporary, reversible protecting system during the
polymer conjugation process. Surfactants have been
shown to be effective in selectively discouraging
polymer aggregates while allowing lysine-based or
10   amino terminal-based conjugation to proceed.

           The present poly(ethylene glycol)-modified hGH or
agonist variant thereof has a more enduring
pharmacological effect, which may be possibly
attributed to its prolonged half-life *in vivo*.

15   Furthermore, the present poly(ethylene glycol)-
modified hGH or agonist variant thereof may be useful
for the treatment of hypo pituitary dwarfism (GHD),
Adult Growth Hormone Deficiency, Turner's syndrome,
growth failure in children who were born short for
20   gestational age (SGA), Prader-Willi syndrome (PWS),
chronic renal insufficiency (CRI), Aids wasting, and
Aging.

           The present poly(ethylene glycol)-modified hGH or
agonist variant thereof may be formulated into
25   pharmaceuticals containing also a pharmaceutically
acceptable diluent, an agent for preparing an isotonic
solution, a pH-conditioner and the like in order to
administer them into a patient.

           The above pharmaceuticals may be administered
30   subcutaneously, intramuscularly, intravenously,
pulmonary, intradermally, or orally, depending on a
purpose of treatment. A dose may be also based on the
kind and condition of the disorder of a patient to be
treated, being normally between 0.1 mg and 5 mg by

injection and between 0.1 mg and 50 mg in an oral
administration for an adult

The polymeric substances included are also
preferably water-soluble at room temperature. A non-
5   limiting list of such polymers include poly(alkylene
oxide) homopolymers such as poly(ethylene glycol) or
poly(propylene glycols), poly(oxyethylenated polyols),
copolymers thereof and block copolymers thereof,
provided that the water solubility of the block
10   copolymers is maintained.

As an alternative to PEG-based polymers,
effectively non-antigenic materials such as dextran,
poly(vinyl pyrrolidones), poly(acrylamides),
poly(vinyl alcohols), carbohydrate-based polymers, and
15   the like can be used. Indeed, the activation of α- and
ω-terminal groups of these polymeric substances can be
effected in fashions similar to that used to convert
poly(alkylene oxides) and thus will be apparent to
those of ordinary skill. Those of ordinary skill in
20   the art will realize that the foregoing list is merely
illustrative and that all polymer materials having the
qualities described herein are contemplated. For
purposes of the present invention, "effectively non-
antigenic" means all materials understood in the art
25   as being nontoxic and not eliciting an appreciable
immunogenic response in mammals.

Definitions

30   The following is a list of abbreviations and the
corresponding meanings as used interchangeably herein:

g   gram(s)
mg   milligram(s)
ml or mL   milliliter(s)

RT	room temperature
PEG	poly (ethylene glycol)

The complete content of all publications,
5   patents, and patent applications cited in this
disclosure are herein incorporated by reference as if
each individual publication, patent, or patent
application were specifically and individually
indicated to be incorporated by reference.

10   Although the foregoing invention has been
described in some detail by way of illustration and
example for the purposes of clarity of understanding,
it will be readily apparent to one skilled in the art
in light of the teachings of this invention that

15   changes and modifications can be made without
departing from the spirit and scope of the present
invention. The following examples ·are provided for
exemplification purposes only and are not intended to
limit the scope of the invention, which has been

20   described in broad terms above.

In the following examples, the hGH is that of SEQ
ID NO:1. It is understood that other members of the
hGH or agonist variant thereof family of polypeptides
could also be pegylated in a similar manner as

25   exemplified in the subsequent examples.

All references, patents or applications cited
herein are incorporated by reference in their entirety
as if written herein.

30

The present invention will be further illustrated
by referring to the following examples, which however,
are not to be construed as limiting the scope of the
present invention.

35

EXAMPLE 1

Straight Chain 20,000 MW PEG-ALD hGH
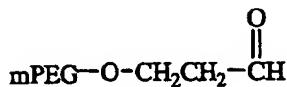
$$mPEG\text{--}O\text{-}CH_2CH_2\text{--}\overset{\overset{\displaystyle O}{\displaystyle \|}}{C}H$$

5

This example demonstrates a method for generation of
substantially homogeneous preparations of N-terminally
monopegylated hGH by reductive alkylation. Methoxy-
10   linear PEG-propionaldehyde reagent of approximately
20,000 MW (Shearwater Corp.) was selectively coupled
via reductive amination to the N-terminus of hGH by
taking advantage of the difference in the relative pK_a
value of the primary amine at the N-terminus versus
15   pK_a values of primary amines at the ε-amino position
of lysine residues. hGH protein dissolved at 10 mg/mL
in 25 mM MES (Sigma Chemical, St. Louis, MO) pH 6.0,
25 mM Hepes (Sigma Chemical, St. Louis, MO) pH 7.0, or·
in 10 mM Sodium Acetate (Sigma Chemical, St. Louis,
20   MO) pH 4.5, was reacted with Methoxy-PEG-
propionaldehyde, M-PEG-ALD, (Shearwater Corp.,
Huntsville, AL) by addition of M-PEG-ALD to yield a
relative PEG:hGH molar ratio of 0.1:0.7 per amine
(optionally 8% acetonitrile may also be added).
25   Reactions were catalyzed by addition of stock 1M
NaCNBH₄ (Sigma Chemical, St. Louis, MO), dissolved in
H_2O, to a final concentration of 10-50 mM. Reactions
were carried out in the dark at 4°C to RT for 18-24
hours. Reactions were stopped by addition of 1 M Tris
30   (Sigma Chemical, St. Louis, MO) ~pH 7.6 to a final
Tris concentration of 50 mM or diluted into
appropriate buffer for immediate purification.

EXAMPLE 2

32

Straight Chain 30,000 MW PEG-ALD hGH

5   Methoxy-linear 30,000 MW PEG-propionaldehyde reagent
      (Shearwater Corp.) was coupled to the N-terminus of
   hGH using the procedure described for Example 1.

                              EXAMPLE 3

10   Straight chain 5,000 MW PEG-ALD hGH

      Methoxy-linear 5,000 MW PEG-propionaldehyde reagent
      (Fluka) was coupled to the N-terminus of hGH using the
      procedure described for Example 1.

15

                              EXAMPLE 4

      Branched chain 40,000 MW PEG-ALD hGH

20
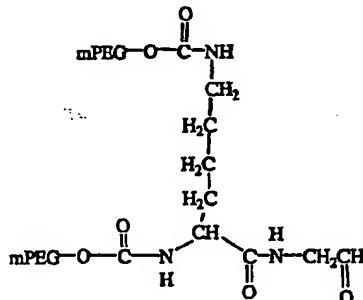
      Methoxy-branched 40,000 MW PEG-aldehyde (PEG2-ALD)
      reagent (Shearwater Corp.) was coupled to the N-
      terminus of hGH using the procedure described for
25   Example 1.

                              EXAMPLE 5

      Branched chain 20,000 MW PEG-ALD hGH

Methoxy-branched 20,000 MW PEG-aldehyde (PEG2-ALD)
reagent (Shearwater Corp.) was coupled to the N-
terminus of hGH using the procedure described for
5   Example 1 using PEG to hGH molar ratios of 0.1-0.5 per
amine.

EXAMPLE 6

10   Straight chain 30,000 MW SPA-PEG hGH

$$mPEG\text{-}O\text{-}CH_2CH_2\overset{\overset{\displaystyle O}{\|}}{C}O\text{---}N$$
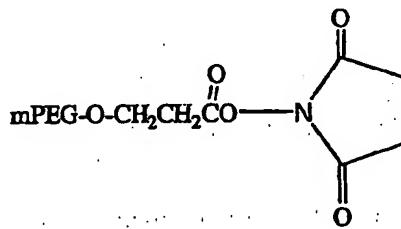
This example demonstrates a method for generation of
15   substantially homogeneous preparations of mono-
pegylated hGH using N-hydroxysuccinimidyl (NHS) active
esters. hGH protein stock solution was dissolved at 10
mg/mL in 0.25 M HEPES buffer, pH 7.2 (optionally 8%
acetonitrile may also be added). The solution was
20   then reacted with Methoxy-PEG-succinimidyl propionate
(SPA-PEG) by addition of SPA-PEG to yield a relative
PEG:hGH molar ratio of 0.1-0.65 per amine. Reactions
were carried out at 4°C to RT for from 5 minutes to I
hour. Reactions were stopped by lowering the pH to 4.0
25   with 0.1 N acetic acid or by adding a 5X molar excess
of Tris HCl.

EXAMPLE 7

30   Straight chain 20,000 MW SPA-PEG hGH

Straight chain 20,000 MW SPA-PEG reagent (Shearwater
Corp.) was coupled to the N-terminus of hGH using the
procedure described for Example 6

5

<center>EXAMPLE 8</center>

Straight chain 3,400 MW Biotin-SPA-PEG-hGH

10
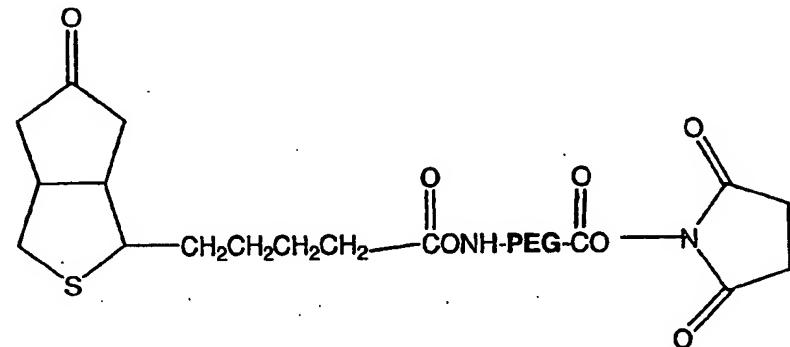
3,400 MW Biotin-PEG-CO_2-NHS reagent (Shearwater Corp.)
is coupled to hGH using the procedure described for
Example 6.

15

<center>EXAMPLE 9</center>

Branched 10,000 MW NHS-PEG-hGH

<center>35</center>
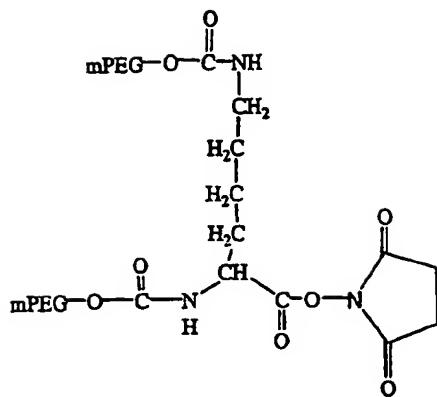
10,000 MW branched PEG2-NHS (Shearwater Corp.) is coupled to hGH using the procedure described for

5   Example 6.

                          EXAMPLE 10

Branched 20,000 MW NHS-PEG-hGH

10

20,000 MW branched PEG2-NHS (Shearwater Corp.) is coupled to hGH using the procedure described for Example 6.

15   EXAMPLE 111

Branched 40,000 MW NHS-PEG-hGH

40,000 MW branched PEG2-NHS (Shearwater Corp.) was
20   coupled to hGH using the procedure described for Example 6.

                          EXAMPLE 12

25   Straight chain 20,000 MW PEG-BTC-hGH
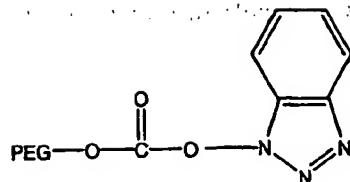
20,000 MW PEG-BTC (Shearwater Corp.) is coupled to hGH using the procedure described for Example 6. This
5   example demonstrates a method for generation of substantially homogeneous preparations of pegylated hGH using benzotriazole carbonate derivatives of PEG.

EXAMPLE 13

10

Straight chain 5,000 MW PEG-SS-hGH
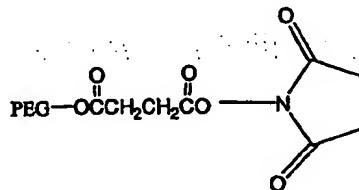
15   5,000 MW succinimidyl succinate-PEG (SS-PEG)(Shearwater Corp.) is coupled to hGH using the procedure described for Example 6. This example demonstrates a method for generation of substantially homogeneous preparations of pegylated hGH using a
20   hydrolyzable linkage.

EXAMPLE 14

Straight chain 20,000 MW PEG-CM-HBA-hGH

25

$$PEG\!-\!OCH_2COCHCH_2CO\!-\!N$$
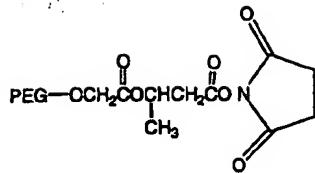
20,000 MW carboxymethyl hydroxybutyric acid-PEG (CM-HBA-PEG)(Shearwater Corp.) was coupled to hGH using

5   the procedure described for Example 6. This example
demonstrates a method for generation of substantially
homogeneous preparations of pegylated hGH using a
hydrolyzable linkage.

10   EXAMPLE 15

Straight chain 2-4x 5,000 MW PEG-CM-HBA-hGH

5,000 MW PEG-CM-HBA (Shearwater Corp.) was coupled to

15   hGH using the procedure described for Example 13.

                            EXAMPLE 16

Straight chain 20,000 MW HZ-PEG hGH

20

                   $PEG\!-\!OCH_2CONHNH_2$

This example demonstrates a method for generation of
substantially homogeneous preparations of pegylated

25   hGH using 20,000 MW methoxy-PEG-hydrazide, HZ-PEG
(Shearwater Corp.). hGH protein stock solution was
dissolved at 10 mg/mL in 10 mM MES, pH 4.0. The
solution was then reacted with HZ-PEG by addition of
solid to yield a relative PEG:hGH molar ratio of 0.1-

30   5.0 per carboxyl group. Reactions were catalyzed with
carbodiimide (EDC, EOAC, EDEC) at a final
concentration of 2 mM to 4 mM. Reactions were carried

38

out at 4°C for 2 hours to overnight or room
temperature from 10 minutes to overnight. Reactions
were stopped by Removing the unconjugated PEG and the
carbodiimide by purification on cation exchange.

5

EXAMPLES 17

Multi-pegylated species

10   Modified hGHs having two or more PEGs (multi-
pegylated) attached were also obtained from Examples 1
and 4 and were separated from the mono-pegylated
species using anion exchange chromatography. Modified
hGHs having two or more PEGs (multi-pegylated)
15   attached are also separated from mono-PEGylated
species using cation exchange chromatography.
Modified hGHs having two or more PEGs (multi-
pegylated) attached are also obtained in examples
2,3,5-13 and are purified in similar fashion to
20   examples 1 and 4.

EXAMPLE 18

Purification of Pegylated hGH

25

Pegylated hGH species were purified from the reaction
mixture to >95% (SEC analysis) using a single ion
exchange chromatography step

30   *Anion exchange chromatography*
The PEG hGH species were purified from the reaction
mixture to >95% (SEC analysis) using a single anion
exchange chromatography step. Mono-pegylated hGH was
purified from unmodified hGH and multi-pegylated hGH
35   species using anion exchange chromatography. A

39

typical 20K aldehyde hGH reaction mixture (5-100 mg
protein), as described above, was purified on a Q-
Sepharose Hitrap column (1 or 5 mL)(Amersham Pharmacia
Biotech, Piscataway, NJ) or Q-Sepharose fast flow

5   column (26/20, 70 mL bed volume)(Amersham Pharmacia
Biotech, Piscataway, NJ) equilibrated in 25 mM HEPES,
pH 7.3 (Buffer A). The reaction mixture was diluted
5-10X with buffer A and loaded onto the column at a
flow rate of 2.5 mL/min. The column was washed with 8

10   column volumes of buffer A. Subsequently, the various
hGH species were eluted from the column in 80-100
column volumes of Buffer A and a linear NaCl gradient
of 0-100 mM. The eluant was monitored by absorbance
at 280 nm (A_{280}) and 5 mL fractions were collected.

15   Fractions were pooled as to extent of pegylation,
e.g., mono, di, tri etc. (as assessed in example 15).
The pool was then concentrated to 0.5-5 mg/mL in a
Centriprep YM10   concentrator (Amicon, Technology
Corporation, Northborough, MA). Protein concentration

20   of pool was determined by A_{280} using an extinction
coefficient of 0.78. Total yield of purified mono 20
K PEG-aldehyde hGH from this process was 25-30 %.

Cation Exchange Chromatography

25   Cation exchange chromatography is carried out on an SP
Sepharose high performance column (Pharmacia XK 26/20,
70 ml bed volume) equilibrated in 10 mM sodium acetate
pH 4.0 (Buffer B). The reaction mixture is diluted
10X with buffer B and loaded onto the column at a flow

30   rate of 5 mL/min. Next the column is washed with 5
column volumes of buffer B, followed by 5 column
volumes of 12% buffer C (10 mM acetate pH 4.5, 1 M
NaCl). Subsequently, the PEG-hGH species is eluted
from the column with a linear gradient of 12 to 27%

35   buffer C in 20 column volumes. The eluant is

monitored at 280 nm and 10 mL fractions are collected.
Fractions are pooled according to extent of pegylation
(mono, di, tri etc.), exchanged into 10 mM acetate pH
4.5 buffer and concentrated to 1-5 mg/mL in a stirred
5   cell fitted with an Amicon YM10 membrane. Protein
concentration of pool is determined by A280 nm using
an extinction coefficient of 0.78. Total yield of
monopegylated hGH from this process is 10 to 50%.

10   EXAMPLE 19

Biochemical Characterization

The purified pegylated hGH pools were characterized by
15 reducing and non-reducing SDS-PAGE, non-denaturing and
denaturing Size Exclusion Chromatography, analytical
Anion Exchange Chromatography, N-terminal Sequencing,
Hydrophobic Interaction Chromatography, and Reversed
Phase HPLC.
20

Size Exclusion High Performance Liquid Chromatography
(SEC-HPLC)

Non-denaturing SEC-HPLC
25 The reaction of Methoxy-PEG of various attachment
chemistries, sizes, linkers, and geometries with hGH,
anion exchange purification pools and final purified
products were assessed using non-denaturing SEC-HPLC.
Analytical non-denaturing SEC-HPLC was carried out
30 using a Tosohaas G4000PWXL column, 7.8 mm X 30 cm,
(Tosohaas Amersham Bioscience, Piscataway, NJ) or
Superdex 200 (Amersham Bioscience, Piscataway, NJ) in
20 mM Phosphate pH 7.2, 150 mM NaCl at a flow rate of
0.5 mL/minute. PEGylation greatly increases the
35 hydrodynamic volume of the protein resulting in a

shift to an earlier retention time. New species were
observed in the PEG aldehyde hGH reaction mixtures
along with unmodified hGH. These PEGylated and non-
PEGylated species were separated on Q-Sepharose
5   chromatography, and the resultant purified mono PEG-
Aldehyde hGH species were subsequently shown to elute
as a single peak on non-denaturing SEC (> 95% purity).
The Q-Sepharose chromatography step effectively
removed free PEG, hGH, and multi PEGylated hGH species
10 from the mono-Pegylated hGH. Non-denaturing SEC-HPLC
demonstrated that the effective size of the various
PEGylated-hGH was much greater than their respective
theoretical molecular weights (Table 1)

15 Table 1
Size Exclusion Chromatography (SEC)

	MW (Theoretical)	Size (SEC)
hGH	22,000	21,000
4-6x5K PEG-SPA GH	47,000	128,000
2-4x5K PEG-CMHBA (NHS) GH	37,000	71,000
20K PEG-ALD GH	42,000	120,000
20K Branched PEG-ALD GH	42,000	114,000
20K PEG-CMHBA (NHS) GH	42,000	115,000
20k PEG-Hydrazide GH	42,000	125,000
2x20K PEG-ALD GH	62,000	250,000
30K PEG-ALD GH	52,000	231,000
30K PEG-SPA GH	52,000	183,000
2x30K PEG-SPA GH	82,000	569,000
40K Branched PEG-ALD GH	62,000	330,000
40K Branched PEG-NHS GH	62,000	253,000

20

Denaturing SEC-HPLC

The reaction of the various Methoxy-PEGs with hGH,
anion exchange purification, and final purified
products were assessed using denaturing SEC-HPLC.

5   Analytical denaturing SEC-HPLC was carried out using a
Tosohaas 3000SWXL column 7.8 mm X 30cm (Tosohaas
Pharmacia Biotech, Piscataway, NJ) in 100 mM Phosphate
pH 6.8, 0.1% SDS at a flow rate of 0.8 mL/minute.
PEGylation greatly increases the hydrodynamic volume

10   of the protein resulting in a shift to an earlier
retention time.   New species were observed in the 20K
PEG aldehyde hGH reaction mixture along with
unmodified hGH. These PEGylated and non-PEGylated
species were separated on Q-Sepharose chromatography,

15   and the resultant purified mono 20K PEG-Aldehyde hGH
was subsequently shown to elute as a single peak on
denaturing SEC(> 95% purity). The Q-Sepharose
chromatography step effectively removed free PEG, hGH,
and multi PEGylated hGH species from the mono-

20   Pegylated hGH.

SDS PAGE/PVDF transfer

SDS-PAGE was used to assess the reaction of the
25   various PEG reagents with hGH and the purified final
products. Examples of this technique are shown with
mono 20K linear and branched 20K and 40K PEG aldehyde,
and 4X6 5K SPA PEG. (Figures 1 & 2). SDS-PAGE was
carried out on 1 mm thick 10-20% Tris tricine gels

30   (Invitrogen, Carlsbad, CA) under reducing and non-
reducing conditions and stained using a Novex
Colloidal Coomassie™ G-250 staining kit (Invitrogen,
Carlsbad, CA). Purified mono PEG-aldehyde hGH species
migrate as one major band on SDS-PAGE. Bands were

35   blotted onto PVDF membrane for subsequent N-terminal

sequence identification.

Analytical anion exchange HPLC

5   Analytical anion exchange HPLC was used to assess the
    reaction of various mPEGs with hGH, anion exchange
    purification fractions and final purified products.
    Analytical anion exchange HPLC was carried out using a
    Tosohaas Q5PW or DEAE-PW anion exchange column, 7.5 mm
10 x 75 mm (Tosohaas Pharmacia Biotech, Piscataway, NJ)
    in 50 mM Tris ph 8.6 at a flow rate of 1 mL/min.
    Samples were eluted with a linear gradient of 5-200 mM
    NaCl.

15 *Reversed phase HPLC (RP-HPLC)*

    PEG-GH reaction mixtures and purified PEGylated
    products were analyzed by RP HPLC to elucidate hGH
    species, mono and multiply PEGylated hGH species, and,
20 to monitor oxidized hGH forms, as well as, PEG hGH
    isoforms having a single PEG linked at different sites
    (e.g. N-terminus vs Lysine ε-amino groups). RP-HPLC
    was carried out utilizing a Zorbax SB-CN 150 or 250 mm
    x 4.6 mm (3.5 mm or 5 mm) reversed phase HPLC column.
25 Experiments were conducted at ambient temperature on a
    typical load of 10 mg of protein per sample. Buffer A
    is 0.1% triflouroacetic acid in water; Buffer B is
    0.1% trifluoroacetic acid in acetonitrile. The
    gradient, which results in a ½% increase in B per
30 minute, is as follows:

Step	Time	Flow	%A	%B	Step
0	0	1	60	40	0
1	3	1	60	40	0
2	20	1	50	50	1
3	2	1	60	40	1
4	6	1	60	40	0

N-terminal Sequence and Peptide Mapping

5 Automated Edman degradation chemistry was used to
determine the NH2-terminal protein sequence. An
Applied Biosystems Model 494 Procise sequencer (Perkin
Elmer, Wellesley, MA) was employed for the
degradation. The respective PTH-AA derivatives were
10 identified by RP-HPLC analysis in an on-line fashion
employing an Applied Biosystems Model 140C PTH
analyzer fitted with a Perkin Elmer/Brownlee 2.1 mm
i.d. PTH-C18 column. 20K linear and 20 and 40K
branched PEG-ALD hGH Protein bands transferred to PVDF
15 membranes or solutions of purified 20K linear and
branched 20 and 40K PEG-ALD hGH were sequenced.
Purified 20K linear PEG-hGH yielded a major signal
(approximately 88% yield) was observed that had the
expected sequence for hGH except for the absence of
20 the N-terminal amino acid. This result is as expected
for a protein N-terminally PEGylated via the aldehyde
chemistry. The residue of the first cycle is
unrecoverable due to the attached PEG moiety. A minor
signal (approximately 12% yield) had the correct N-
25 terminal amino acid sequence. Considering that the
peak collected off the RP-HPLC is 100% PEGylated,
these data suggest that approximately 88% of the PEG
modification is at the N-terminus with remainder
apparently linked to one of several possible lysine

residues.

Tryptic digests were performed at a concentration
of 1 mg/mL and, typically, 50 ug of material is used
5   per digest. Trypsin was added such that the trypsin to
PEG-hGH ratio was 1:30 (w/w). Tris buffer was present
at 30 mM, pH 7.5. Samples were incubated at room
temperature for 16 ± 0.5 hours. Reactions were
quenched by the addition of 50 μL of 1N HCl per mL of
10 digestion solution. Samples were diluted, prior to
placing the samples in the autosampler, to a final
concentration of 0.25 mg/ml in 6.25 % acetonitrile.
Acetonitrile is added first (to 19.8% acetonitrile),
mixed gently, and then water is added to final volume
15 (four times the starting volume). Extra digestion
solution may be removed and stored for up to 1 week at
-20°C.

A Waters Alliance 2695 HPLC system was used for
analysis, but other systems should produce similar
20 results. The column used was an Astec C-4 polymeric
25 cm x 4.6 mm column with 5 μm particles.
Experiments were conducted at ambient temperature on a
typical load of 50 μg of protein per sample. Buffer A
is 0.1% trifluoroacetic acid in water; buffer B is
25 0.085% trifluoroacetic acid in acetonitrile. The
gradient is as follows:

Time	A%	B%	C%	D%	Flow	Curve
0.00	0.0	0.0	100.0	0.0	1.000	1
90.00	0.0	0.0	55.0	45.0	1.000	6
90.10	0.0	0.0	0.0	100.0	1.000	6
91.00	0.0	0.0	0.0	100.0	1.000	6
91.10	0.0	0.0	100.0	0.0	1.000	6
95.00	0.0	0.0	100.0	0.0	1.000	6

The column is heated to 40°C using a heat jacket.
Peaks were detected using a Waters 996 PDA detector
collecting data between 210 and 300 nm. The extracted
chromatogram at 214 nm was used for sample analysis to

5   determine the extent of n-terminal Pegylation (loss of
T-1 fragment) as shown in Table 2.

Table 2

Sample	% T-1 present	% T-1 Lost	%T-1 Present compared to control
Aldehyde			
5K ALD	2.0	98.0	7.4
20K	0.0	100.0	0.0
2x20K	0.0	100.0	0.0
30K	1.3	98.7	4.5
40K Branched	1.9	98.1	6.8
NHS			
4-6x5 SPA	1.3	98.7	4.7
2-4X5 CM	0.0	100.0	0.0
20K CM	23.1	76.9	84.1
30K	18.2	81.8	63.9
2x30K	5.7	94.3	19.9
40K branched	20.9	79.1	73.5

10   Example 20

Pharmacodynamic Studies

Efficacy studies in Hypophysectomized rats
Female Sprague Dawley rats, hypophysectomized at

15 Harlan Labs, were prescreened for growth rate for a
period of 7 to 10 days. Subsequently, growth studies
were carried out for 11 days. Rats were divided into
groups of six to eight. Group 1 consisted of rats
given either daily or day 0 and day 6 subcutaneous

20 dose(s) of vehicle. Group 2 were given daily

subcutaneous doses of GH (0.3 mg/kg/dose). Group 3
were given subcutaneous doses of GH on day 0 and day
6(1.8 mg/kg/dose). Group 4 were given subcutaneous
doses of PEG-GHs on day 0,6 (1.8 mg/kg/dose).

5   Hypophysectomized rats were monitored for weight gain
by weighing at least every other day during the study.
Weight gains (average +/- SEM) for 20K PEG-ALD hGH,
20K and 40K branched PEG-ALD hGH, and 4-6x 5PEG-SPA
hGH dosed once a week were similar to those for daily

10   dosing of hGH (Figures 3& 4) Table 3 summarizes total
weight gain (average +/- SEM) at day 11 for once per
week dosing of various Pegylated hGH molecules
relative to daily dosing of hGH.

15   Table 3
     Murine weight gain

Compound	Single Weekly Dose (mpk)	Daily weight gain in grams/day (d0-d11) (Avg. + SEM)	% weight gain relative to daily hGH gain (Avg.)
hGH (un-pegylated)	1.8	0.97 + 0.12	39%
5K Linear PEG-ALD GH	1.8	0.96 + 0.27	36%
20K Linear PEG-ALD GH	1.8	1.99 ± 0.13, 1.43 ± 0.08, 1.7 + 0.10	73%
20K Linear CM-HBA PEG GH	1.8	2.36 + 0.11	99%
20K Linear PEG-HYD GH	1.8	2.62 + 0.22	99%
20K Branched PEG-ALD GH	1.8	2.24 + 0.07	87%
30K Linear PEG-ALD GH	1.8	2.11+ 0.06; 1.85+ 0.14	94%
30K Linear PEG-SPA GH	1.8	2.6 +/- 0.1	117%
40K Branched PEG-ALD GH	1.8	2.57 + 0.08	100%
40K Branched PEG-NHS GH	1.8	2.53 + 0.09	121%
2x 20K PEG-ALD GH	1.8	2.66 + 0.10	128%
4-6x5K SPA-PEG GH	1.8	3.18 + 0.10	124%
2-4x5K CM-HBA-PEG GH	1.8	3.54 + 0.15	134%
2x 30K Linear PEG-SPA GH	1.8	3.1 + 0.1	134%

20   Upon completion of each growth study, animals were
sacrificed and bone (tibia) lengths were analyzed.
Figure 6 shows the change in tibial bone length
(average +/- SEM) at day 11 in response to various

PEG-GH conjugates dosed on day 0 and day 6 or hGH
dosed daily.

IGF-1 levels in hypophysectomized rats

5

Experiments were carried out as above for the weight
gain studies, however blood samples were taken at day
0,1,2,3,4,5 and upon sacrifice of the animals at day
9. IGF-1 levels were determined by ELISA. Figure 7
10 compares increases in serum IGF-1 levels (average +/-
SEM) in hypophysectomized rats following either daily
dosing of hGH or single dose of hGH or day 0, day 6
dosing of Pegylated hGH.

15 *Pharmacokinetic Studies*

Pharmacokinetic studies were conducted in normal,
Sprague-Dawley male rats, mice, and cynomolgus
monkeys. Injections were made, either as a single
20 subcutaneous bolus of 1.8 mg/kg or as a single iv dose
at 1.0 mg/kg GH or PEG-GH in rats and mice, using six
rats and up to 60 mice per group. In cynomolgus
monkeys a 0.18 mg/kg GH or PEG-GH dose was used for
both single subcutaneous bolus and iv, using 2-4
25 monkeys per group. Blood samples were taken over one
to five days as appropriate for assessment of relevant
PK parameters (Table 4). $(t_{1/2})$ = Terminal half life,
(Cl) = clearance, (Tmax) = time to maximum
concentration, Vss = volume distribution (apparent) at
30 steady state, and (Cmax) = maximum concentration GH
and PEG-GH blood levels were monitored at each
sampling using immuno-assay.

hGH Immunoassay

hGH and pegylated hGH protein concentration
levels in rat, mouse, and cynomolgus monkey plasma
were determined using the hGH AutoDELFIA kit
fluorescence immunoassay (PerkinElmer Life Sciences),
5   using the appropriate PEG hGH to generate standard
curve.

Table 4

Species	Parameters	40K Br ALD hGH	40K Br NHS hGH	30K ALD hGH	20K ALD hGH	4-6 x 5K SPA
mouse	dose (mg/kg)	Iv 1.0 sc 1.8	Iv 1.0 sc 1.8	Iv 1.0 sc 1.8	Iv 1.0 sc 1.0	iv 1.0 sc 1.8
	CL (ml/hr/kg)	2.29	2.12	4.43	7.89	4.53
	Vss (ml/kg)	18	16	24	17	51
	$T_{1/2, iv}$ (hr)	4.3	3.8	2.8	1.8	11
	$T_{1/2, sc}$ (hr)	4	6.2	3.7	2.5	9
	Tmax, sc (hr)	11	9	6	3	12
	SC AUC (ug/ml*hr)	682	577	160	31	668
	SC Bioavailability (%)	87	67	39	24	167
rat	dose (mg/kg)	iv 1.0 sc 1.8	Iv 1.0 sc 1.8	iv 1.0 sc 1.8	Iv 1.8 sc 1.8	Iv 1.0 sc 1.8
	CL (ml/hr/kg)	1.36	1.75	5.75	9.9	2.9
	Vss (ml/kg)	19	25	44	33	36
	$T_{1/2, iv}$ (hr)	5.4	5.8	3.6	2.2	24
	$T_{1/2, sc}$ (hr)	5.8	7.1	6.7	2.9	29
	Tmax, sc (hr)	24	22	12	9	20
	SC AUC (ug/ml*hr)	398	344	97	70	249
	SC Bioavailability (%)	30	33	31	39	40
cyno	dose (mg/kg)	Iv 0.18 sc 0.18	Iv 0.18 sc 0.18	Iv 0.18 sc 0.18	Iv 0.18 sc 0.18	Iv 0.18 sc 0.18
	CL (ml/hr/kg)	1.83	0.78	1.94	2.19	0.49
	Vss (ml/kg)	57	20	29	44	25
	$T_{1/2, iv}$ (hr)	21	13.6	14.9	7.3	38
	$T_{1/2, sc}$ (hr)	19	21	12	8.3	35
	Tmax, sc (hr)	22	22	10	8	32
	SC AUC (ug/ml*hr)	100	483	125	38	242
	SC Bioavailability (%)	64	77	97	44	66

WHAT IS CLAIMED IS:

1.   A conjugate comprising at least one water-
soluble polymer molecule covalently attached to at
least one amino acid residue of a biologically active
human growth hormone (hGH) polypeptide or agonist
variant thereof.

2.   The conjugate of claim 1 wherein said hGH
polypeptide comprises the amino acid sequence of SEQ
ID NO:1.

3.   The conjugate of claim 1, or 2, wherein said
polymer is a poly(ethylene oxide) molecule.

4.   The conjugate of claim 3 wherein said
poly(ethylene oxide) molecule is a poly(ethylene
glycol) molecule.

5.   The conjugate of claim 4 wherein the
poly(ethylene glycol) is attached at an amino acid
residue having a free amino group(s), carboxyl
group(s), or sulfhydryl group(s).

6. The conjugate of claim 5 formed using a
activated poly(ethylene glycol).

7.   The conjugate of claim 6 wherein said
activated poly(ethylene glycol) comprises a
functional group.

8.   The conjugate of claim 7 wherein said
attachment is at an amino acid having a free amino
group.

9. The conjugate of claim 8 wherein said functional group is selected from the group consisting of: carbonates, carbonyl imidazole, active esters of carboxylic acids, azlactones, cyclic imide thiones,
5 isocyanates or isothiocyanates, imidates, and aldehydes.

10. The conjugate of claim 9 wherein said functional group is a carbonate or carbonyl imidazole.
10

11. The conjugate of claim 10 wherein said activated poly(ethylene glycol) is selected from the group consisting of:
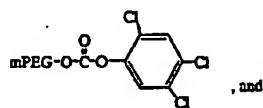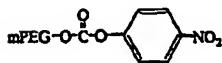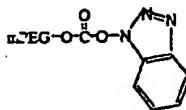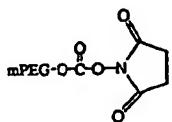
15
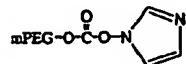
12. The conjugate of claim 11 having the structure

$$\text{mPEG-O-C-N-R}$$

20
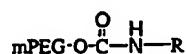
wherein R is a human growth hormone polypeptide.

13. The conjugate of claim 12 wherein said human
growth hormone polypeptide comprises the amino acid
5   sequence of SEQ ID NO:1.

14. The conjugate of claim 9 wherein said
functional group is a cyclic imide thione.

10   15. The conjugate of claim 14 wherein said
activated poly(ethylene glycol) is selected from the
group consisting of:
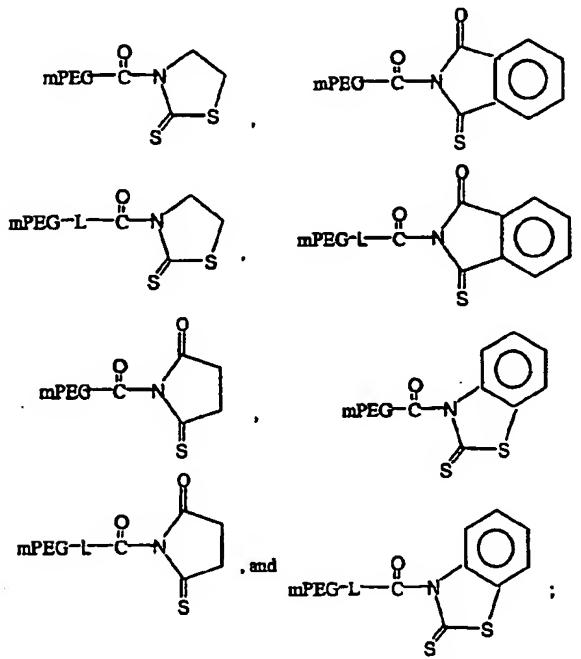
15   wherein L is selected from the group consisting
of: -O-, -NH-, -OCH$_2$-, -NH-CO(CH$_2$)$_n$, -NH-CO(CH$_2$)$_n$O-, -
CO-NH(CH$_2$)$_n$-, -S-, -CO-NH(CH$_2$)$_n$O-, -O(CH$_2$)$_N$O-, -O(CH$_2$)$_n$-,
-SCH$_2$CH$_2$-, AND -NH(CH$_2$)$_n$-.

16. The conjugate of claim 9 wherein said
functional group is a azlactone.

17. The conjugate of claim 16 wherein said
5   activated poly(ethylene glycol) is selected from the
group consisting of:

, and
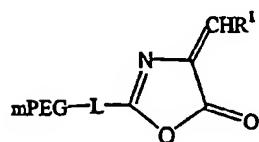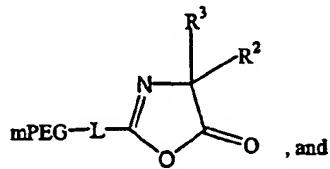
10   wherein
R^1 is selected from the group consisting of
hydrogen, alkyl, cycloalkyl, carbocyclic and
heterocyclic aromatic rings, α, β-unsaturated alkyl;
and
15   R^2 and R^3 are independently selected from
hydrogen, alkyl, aryl, and alkylaryl.

18. The conjugate of claim 9 wherein said
functional group is a isocyanate or isothiocyanate.
20

19. The conjugate of claim 18 wherein said
activated poly(ethylene glycol) is selected from the
group consisting of:

25   mPEG-N=C=O, and mPEG-N=C=S.

55

20. The conjugate of claim 9 wherein said functional group is an aldehyde or aldehyde hydrate.

21. The conjugate of claim 20 wherein said
5 activated poly(ethylene glycol) is selected from the group consisting of:

$$mPEG\text{-}O\text{-}CH_2CHO_2, \quad mPEG\text{-}OCH_2CH_2CH_2\overset{\overset{\displaystyle O}{\|}}{C}H,$$

$$mPEG\text{-}OCH_2CH_2CH_2\overset{\overset{\displaystyle O}{\|}}{C}H, \text{ and } mPEG\text{-}O\text{-}CH_2CHO_2,$$

10

$$mPEG\text{-}OCH_2CH_2CH(OH)_2,$$

, and
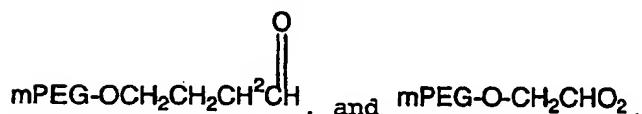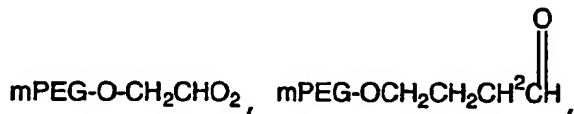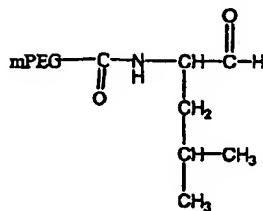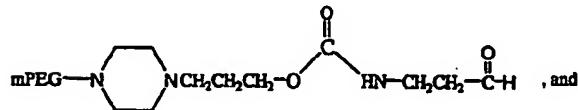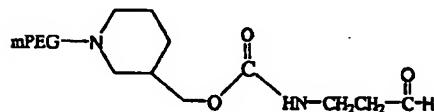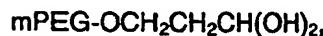
15   22. The conjugate of claim 9 wherein said functional group is an active ester of a carboxylic acid.

23. The conjugate of claim 22 wherein said
activated poly(ethylene glycol) is selected from the
group consisting of:

5
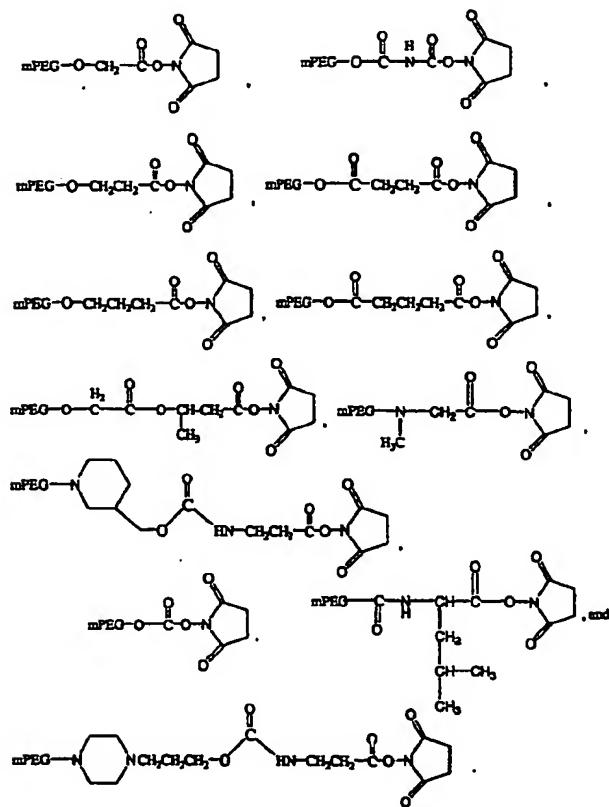
24. The conjugate of claim 23 having the
structure selected from the group consisting of:

mPEG—O—CH$_2$—C(=O)—NH-R   mPEG—O—C(=O)—N(H)—C(=O)—NH-R

mPEG—O—CH$_2$CH$_2$—C(=O)—NH-R ,   mPEG—O—C(=O)—CH$_2$CH$_2$—C(=O)—NH-R ,

mPEG—O—CH$_2$CH$_2$CH$_2$—C(=O)—NH-R ,   mPEG—O—C(=O)—CH$_2$CH$_2$CH$_2$—C(=O)—NH-R ,

mPEG—O—C(H$_2$)—C(=O)—O—CH(CH$_3$)CH$_2$—C(=O)—NH-R ,   mPEG—N(H$_3$C)—CH$_2$—C(=O)—NH-R ,

mPEG—N(piperidine ring)—CH$_2$—O—C(=O)—HN—CH$_2$CH$_2$—C(=O)—NH-R ,

mPEG—O—C(=O)—NH-R ,   mPEG—C(=O)—N(H)—CH—C(=O)—NH-R , and
                                              |
                                              CH$_2$
                                              |
                                              CH—CH$_3$
                                              |
                                              CH$_3$

mPEG—N(piperazine ring)N—CH$_2$CH$_2$CH$_2$—O—C(=O)—HN—CH$_2$CH$_2$—C(=O)—NH-R ;
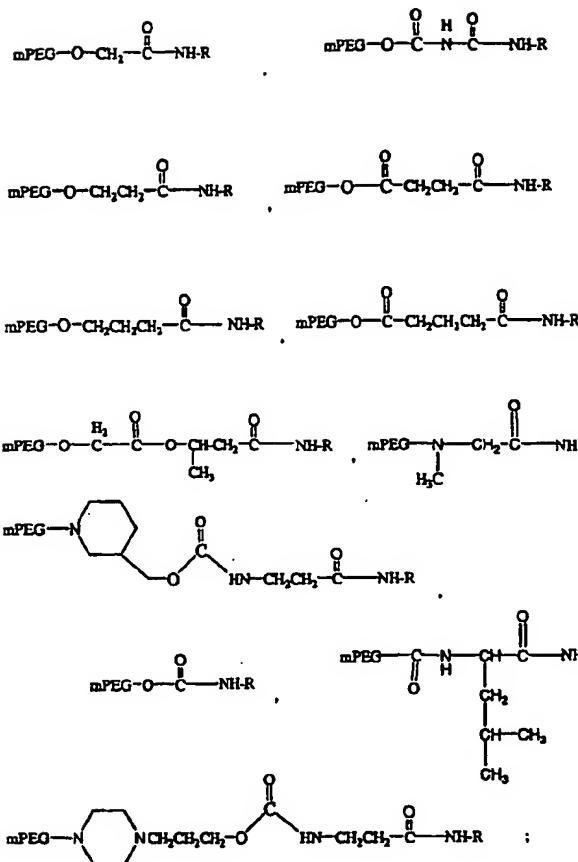
wherein R is a human growth hormone polypeptide.

25.   The conjugate of claim 24 wherein said human
growth hormone polypeptide comprises the amino acid
sequence of SEQ ID NO:1.

26.   The conjugate of claim 9 wherein said
activated poly(ethylene glycol) is selected from the
group consisting of:

mPEG-O-SO$_2$-CH$_2$CF$_3$, and   mPEG—O—(triazine ring with two Cl)
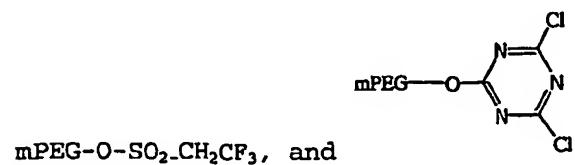
27. The conjugate of claim 26 having the structure selected from the group consisting of:

PEG-NH-R, and
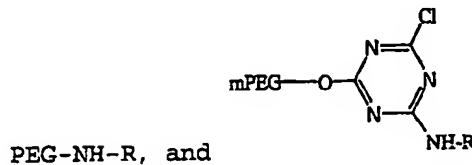
5

wherein R is a human growth hormone polypeptide.

28. The conjugate of claim 27 wherein said human growth hormone polypeptide comprises the amino acid

10 sequence of SEQ ID NO:1.

29. The conjugate of claim 28 wherein said functional group is an imidate.

15   30. The conjugate of claim 9 wherein said activated poly(ethylene glycol) is selected from the group consisting of:

$$mPEG\text{-}NH\text{-}(CH_2)_m\text{-}\overset{\overset{\displaystyle OCH_3}{|}}{C}=NH_2^+Cl^-$$ wherein m is 0 to 20, and

$$mPEG\text{-}NHC(O)\text{-}\overset{\overset{\displaystyle OR^1}{|}}{C}=NH_2^+Cl^-$$ wherein R^1 is alkyl, phenyl, phenylalkyl, and cycloalkyl.
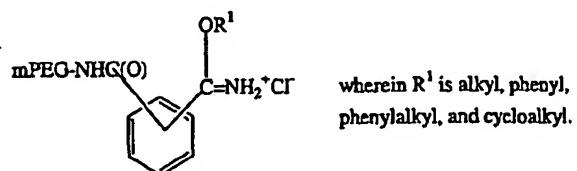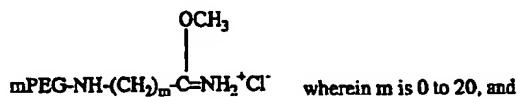
20

31. The conjugate of claim 9 wherein said free amino group is an amino terminal α-amino group.

32. The conjugate of claim 31 wherein said amino
25 terminal α-amino group is on a phenylalanine.

33. The conjugate of claim 8 wherein said attachment is at an amino acid having a free carboxyl group.

34. The conjugate of claim 33 wherein said functional group is selected from the group consisting of: primary amines; hydrazine; and hydrazide functional groups.

35. The conjugate of claim 34 wherein said functional group is selected from the group consisting of:

mPEG-CH2CH2-NH2, mPEG-O-CH2-CO-NH-NH2,
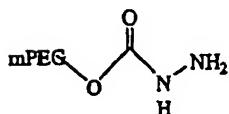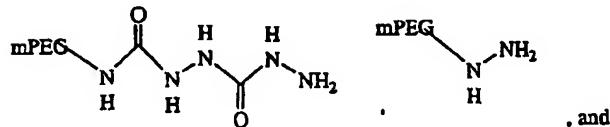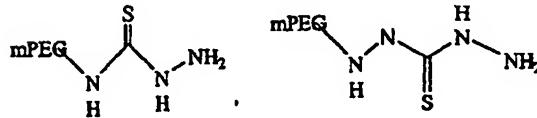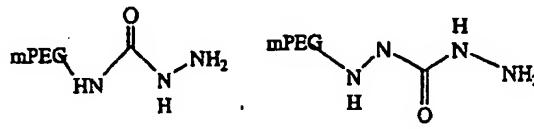
36. The conjugate of claim 8 wherein said attachment is at an amino acid having a free sulfhydryl group.

37. The conjugate of claim 36 wherein said
functional group is selected from the group consisting
of: thiols; maleimides; vinyl sulfones; and phenyl
5   glyoxals.

38. The conjugate of claim 37 wherein said
functional group is selected from the group consisting
of:

10

mPEG—N maleimide structure   mPEG—S(=O)(=O)—C(H)=CH$_2$

mPEG—HN—C(=O)—CH$_2$—I , and   mPEG—S=S—pyridyl
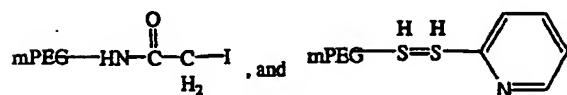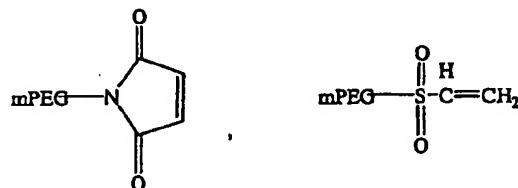
39. The conjugate of claim 38 having the
structure selected from the group consisting of:

15

mPEG—N succinimide S-R structure   mPEG—S(=O)(=O)—CH$_2$CH$_2$-S-R

mPEG—HN—C(=O)—CH$_2$-S-R , and   mPEG—S—S-R
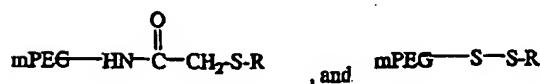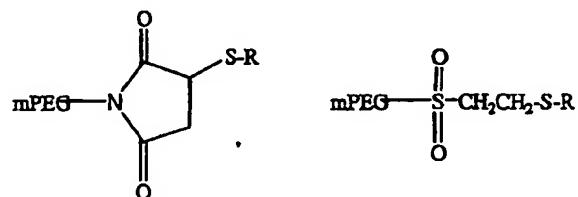
wherein R is a human growth hormone polypeptide.

40. The conjugate of claim 39 wherein said human growth hormone polypeptide comprises the amino acid sequence of SEQ ID NO:1.

41. The conjugate of claim 8 wherein said poly(ethylene glycol) has a molecular weight of between about 0.5 kDa and about 100 kDa.

42. The conjugate of claim 41 wherein said poly(ethylene glycol) has a molecular weight of between about 5 kDa and about 40 kDa.

43. The conjugate of claim 8 wherein said poly(ethylene glycol) is a branched polymer.

44. The conjugate of claim 43 wherein said branched polymer is selected from the group consisting of:
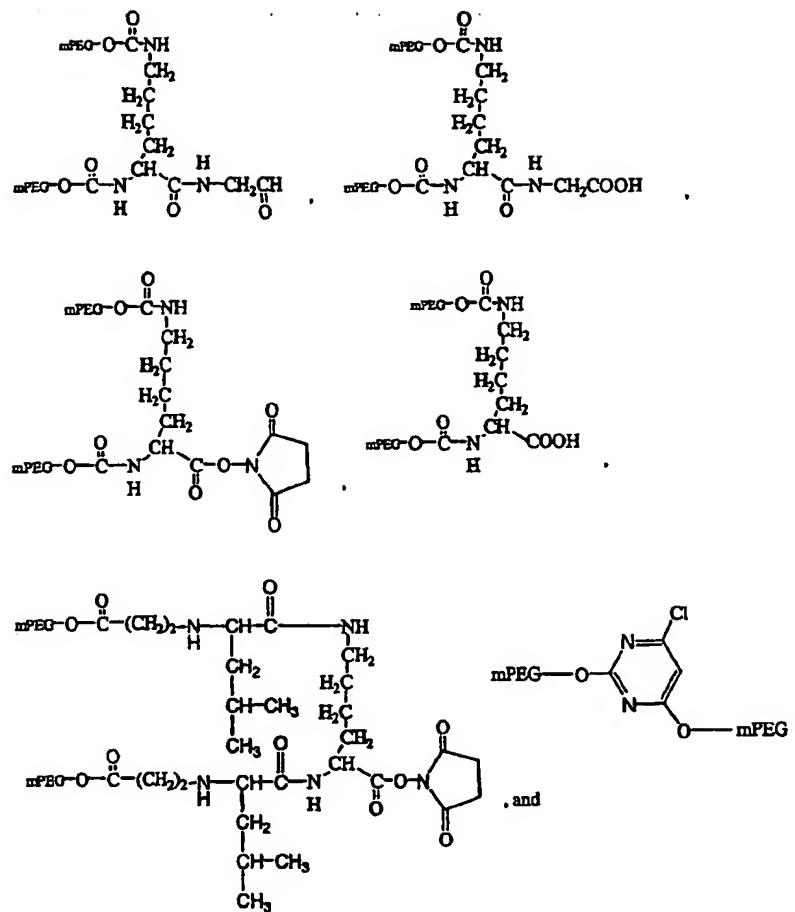
45. A human growth hormone-PEG conjugate having
the structure
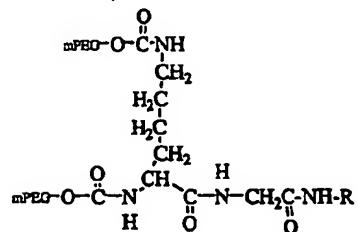
wherein R is a human growth hormone polypeptide.

46. The conjugate of claim 45 wherein said human growth hormone polypeptide comprises the amino acid sequence of SEQ ID NO:1.

5   47. The conjugate of claim 46 wherein at least 80% of said polyethylene glycol is conjugated to the amino-terminal phenylalanine.

48   The conjugate of claim 46 wherein at least
10   90% of said polyethylene glycol is conjugated to the amino-terminal phenylalanine.

49. The conjugate of claim 47 or 48 wherein each mPEG has a molecular weight of about 20 kDa.

15

50. A human growth hormone-PEG conjugate having the structure

20
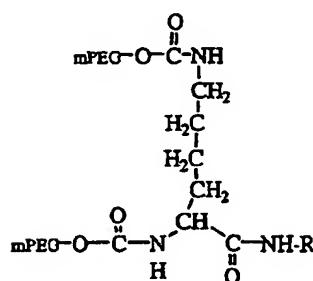
wherein R is a human growth hormone polypeptide.

51. The conjugate of claim 50 wherein said human growth hormone polypeptide comprises the amino acid
25   sequence of SEQ ID NO:1.

52. The conjugate of claim 51 wherein at least 80% of said polyethylene glycol is conjugated to the amino-terminal phenylalanine.

64

53. The conjugate of claim 51 wherein at least 90% of said polyethylene glycol is conjugated to the amino-terminal phenylalanine.

54. The conjugate of claim 52 or 53 wherein each mPEG has a molecular weight of about 20 kDa.

55. A human growth hormone-PEG conjugate having the structure

mPEG-OCH$_2$CH$_2$CH$_2$NH-R
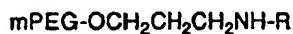
wherein R is a human growth hormone polypeptide.

56. The conjugate of claim 55 wherein said human growth hormone polypeptide comprises the amino acid sequence of SEQ ID NO:1.

57. The conjugate of claim 56 wherein at least 80% of said polyethylene glycol is conjugated to the amino-terminal phenylalanine.

58. The conjugate of claim 56 wherein at least 90% of said polyethylene glycol is conjugated to the amino-terminal phenylalanine.

59. The conjugate of claim 57 or 58 wherein each mPEG has a molecular weight of about 20 kDa.

60. The conjugate of claim 8 wherein said poly(ethylene glycol) is a bifunctional polymer.

61. The conjugate of claim 8 wherein said poly(ethylene glycol) is a prodrug.

62. A composition comprising the hGH of claim 1 and at least one pharmaceutically acceptable carrier.

5   63. A method of treating a patient having a growth or development disorder or comprising administering to said patient a therapeutically effective amount of the hGH conjugate of claim 1.

10   64. The method of claim 63 wherein said growth or development disorder is Growth Hormone Deficiency (GHD).

65. The method of claim 63 wherein said growth
15 or development disorder is Turner's syndrome.

66. The method of claim 63 wherein said growth or development disorder is Chronic Renal Insufficiency.
20

67. The method of claim 63 wherein said growth or development disorder is small for gestational age (SGA).

25   68. A composition comprising the hGH of claim 48 and at least one pharmaceutically acceptable carrier.

69. A method of treating a patient having a growth or development disorder or comprising
30 administering to said patient a therapeutically effective amount of the hGH conjugate of claim 48.

70. The method of claim 69 wherein said growth or development disorder is Growth Hormone Deficiency
35 (GHD).

71. The method of claim 69 wherein said growth or development disorder is Turner's syndrome.

72. The method of claim 69 wherein said growth or development disorder is Chronic Renal Insufficiency.

73. The method of claim 69 wherein said growth or development disorder is small for gestational age (SGA).

74. A composition comprising the hGH of claim 50 or 55 and at least one pharmaceutically acceptable carrier.

75. A method of treating a patient having a growth or development disorder or comprising administering to said patient a therapeutically effective amount of the hGH conjugate of claim 1.

76. The method of claim 75 wherein said growth or development disorder is Growth Hormone Deficiency (GHD).

77. The method of claim 75 wherein said growth or development disorder is Turner's syndrome.

78. The method of claim 75 wherein said growth or development disorder is Chronic Renal Insufficiency.

79. The method of claim 75 wherein said growth or development disorder is small for gestational age (SGA).

Figure 1

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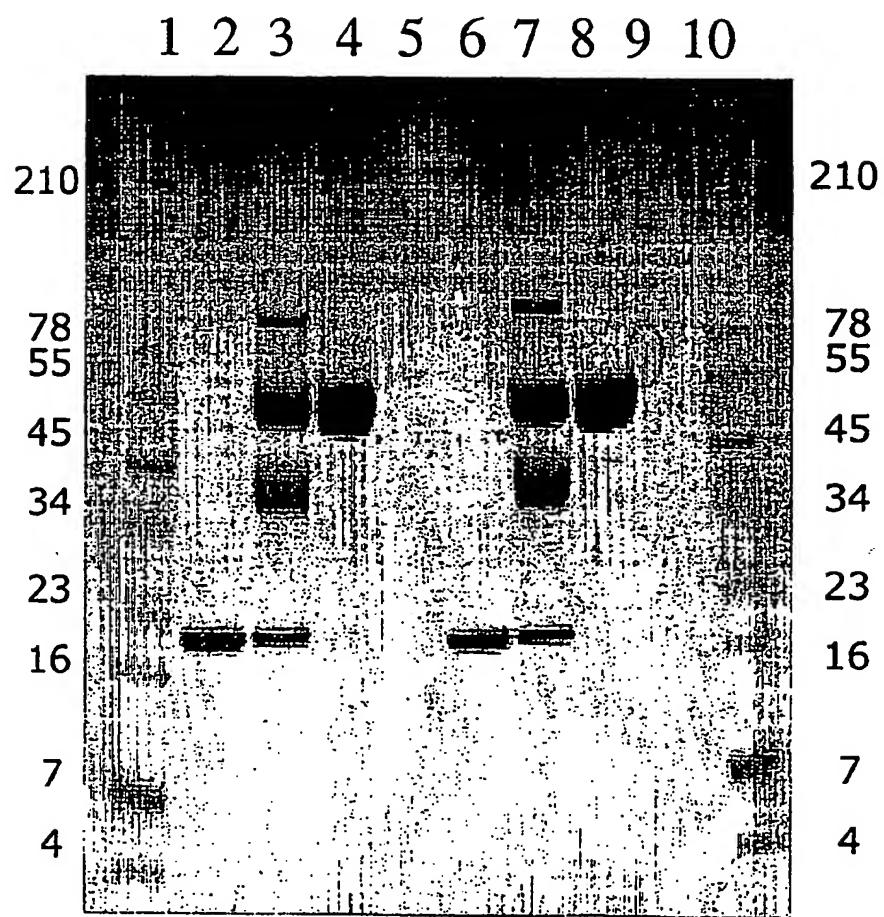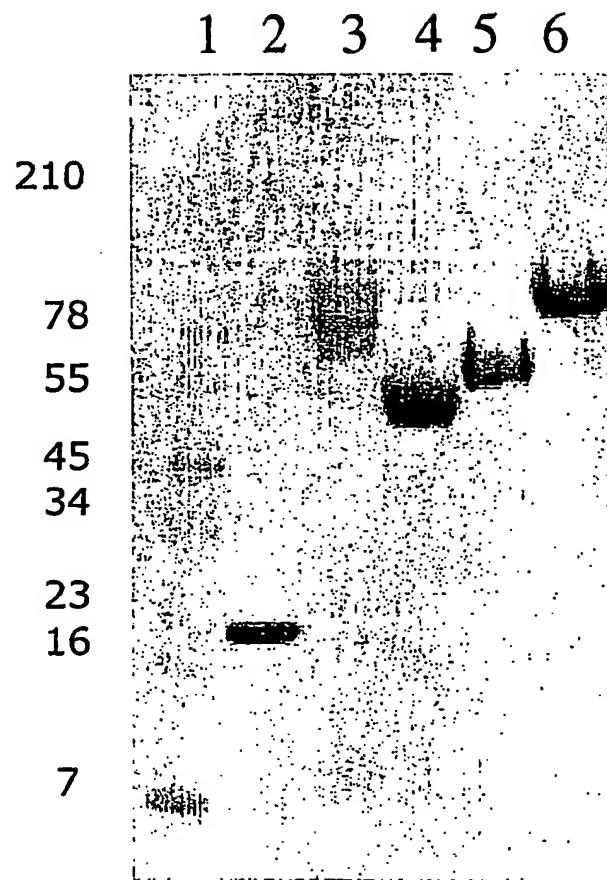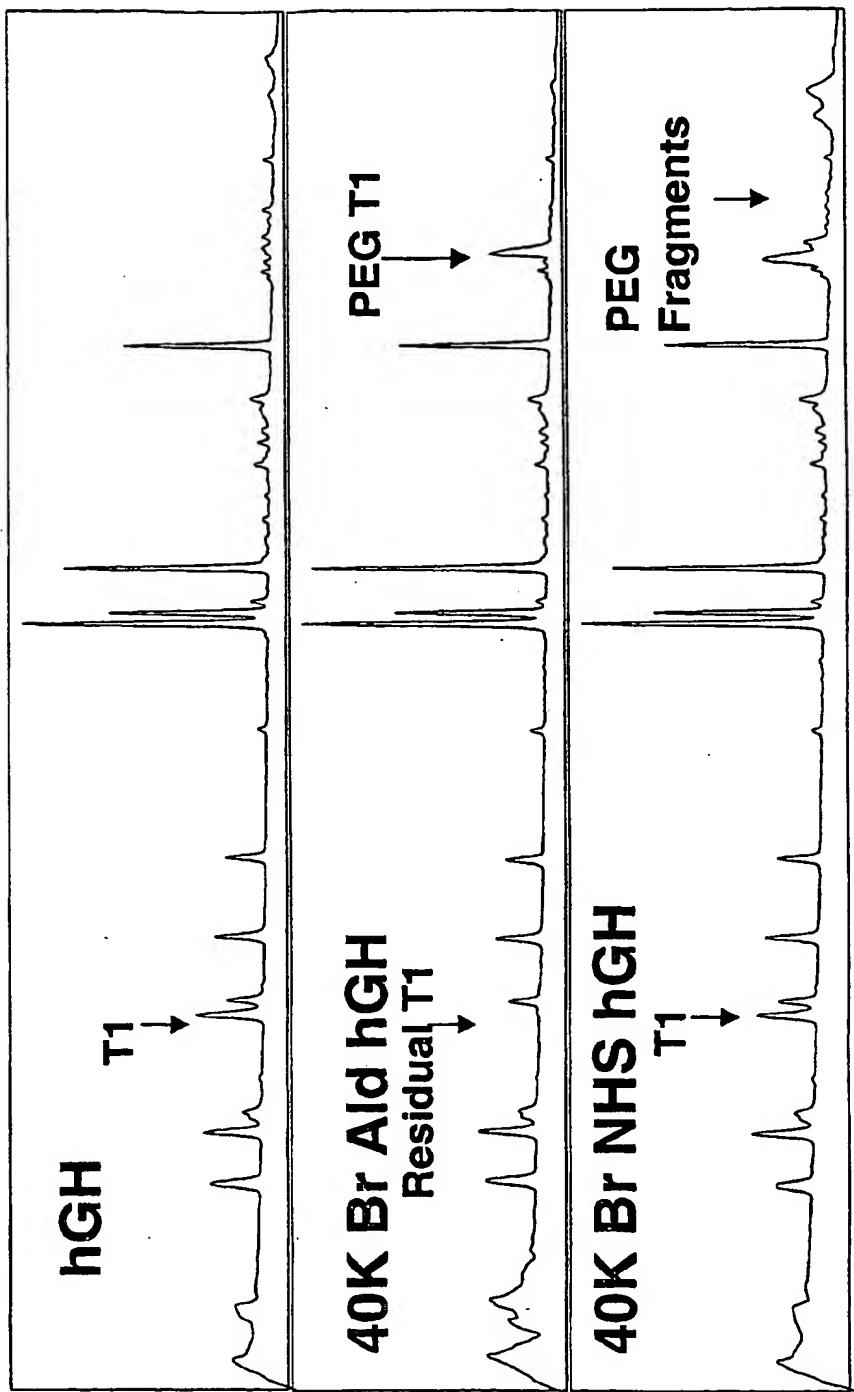
Figure 2

Figure 3

Figure 4
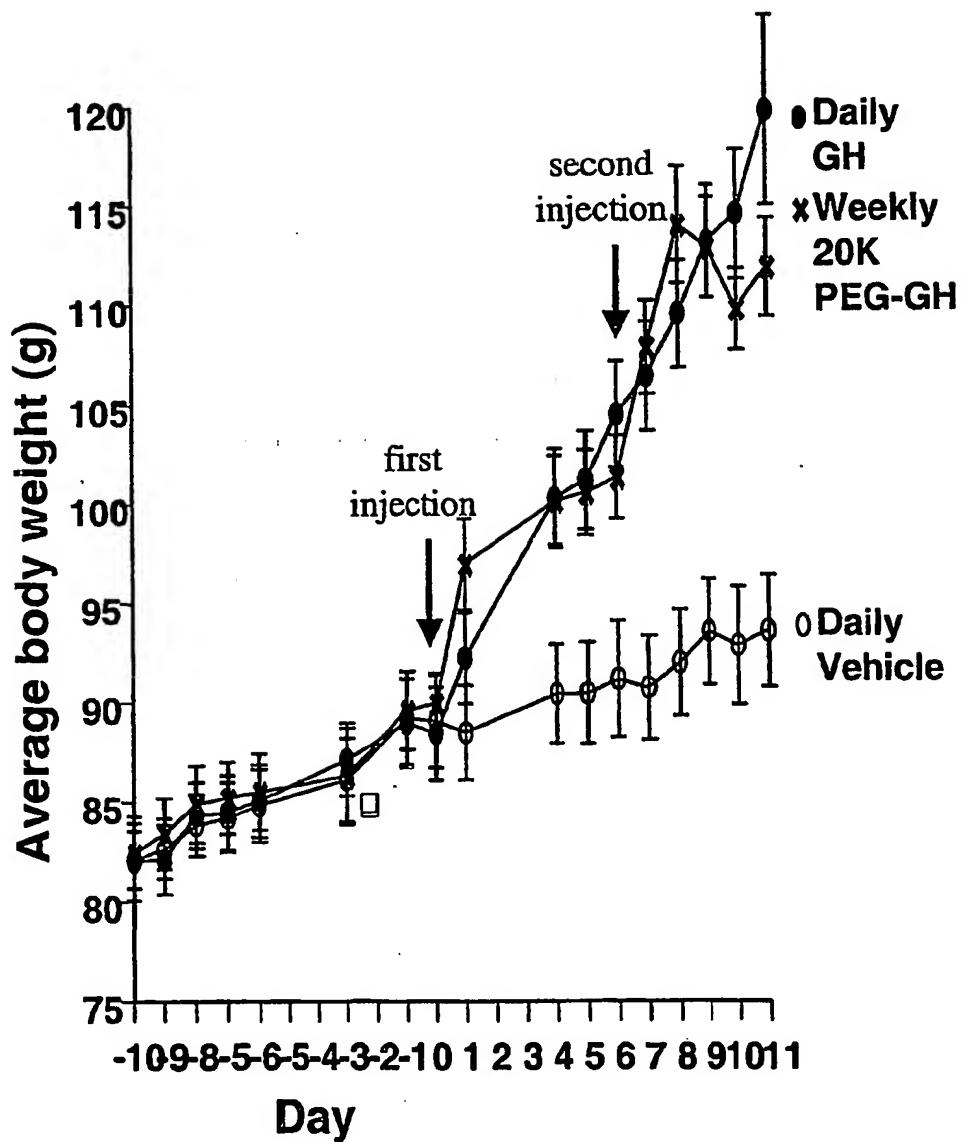
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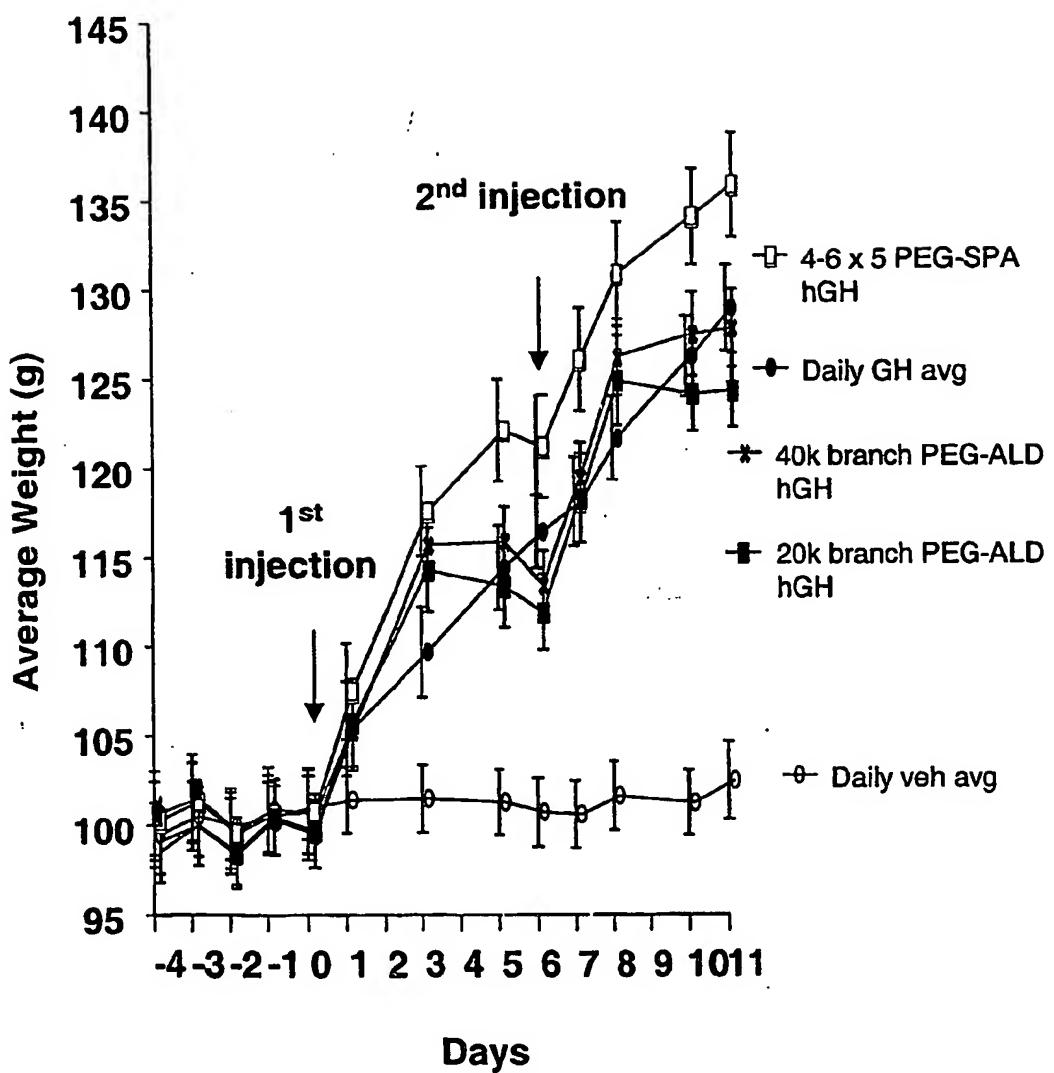
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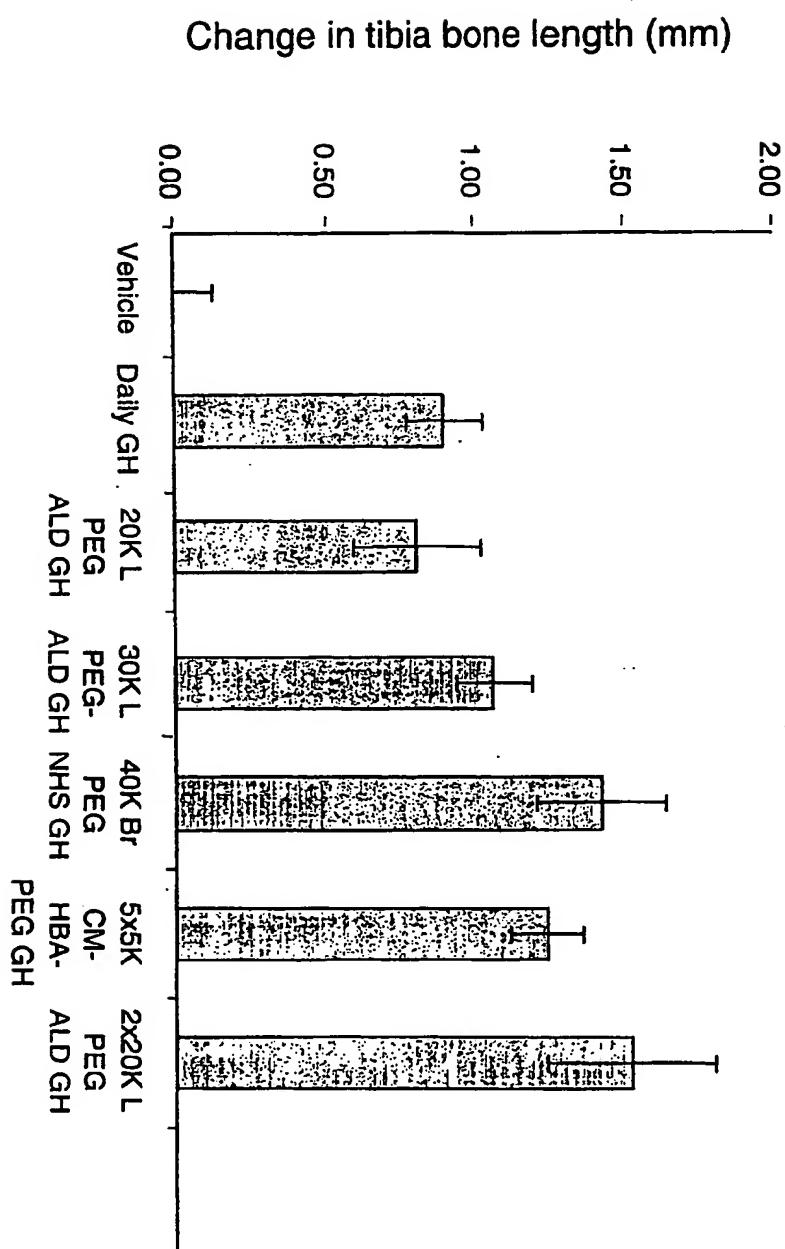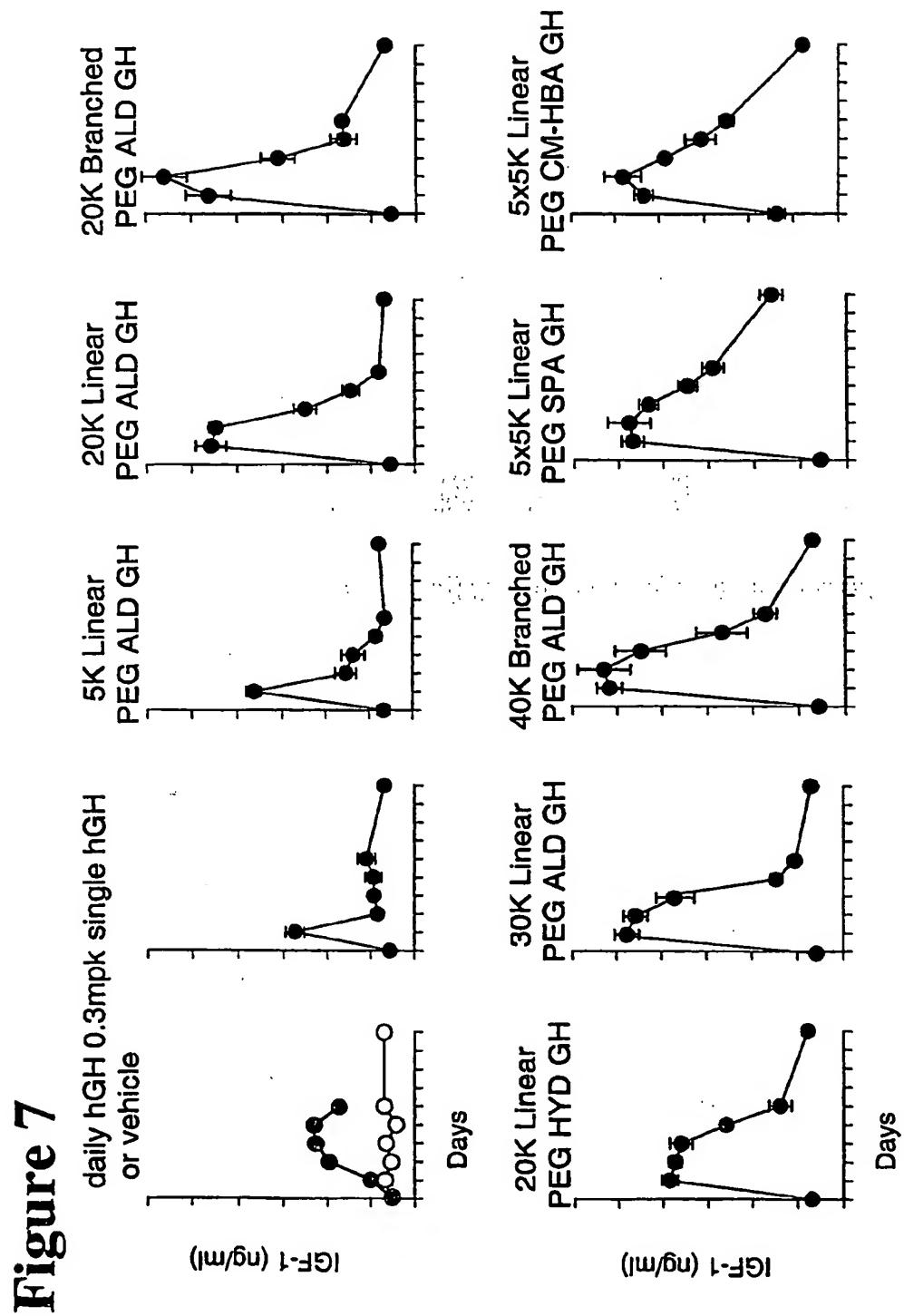
Figure 7

Figure 8
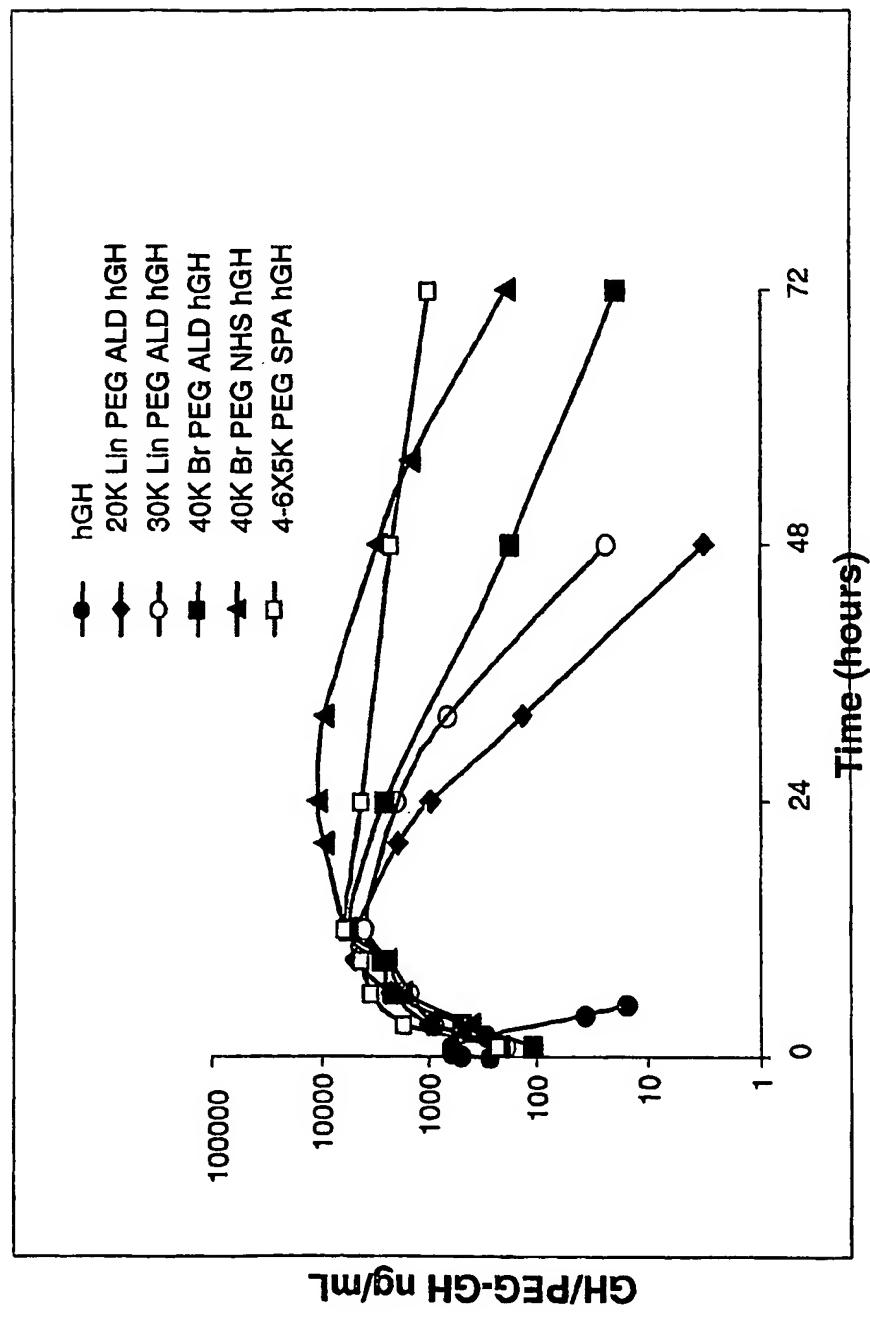
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       Finn, Rory

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